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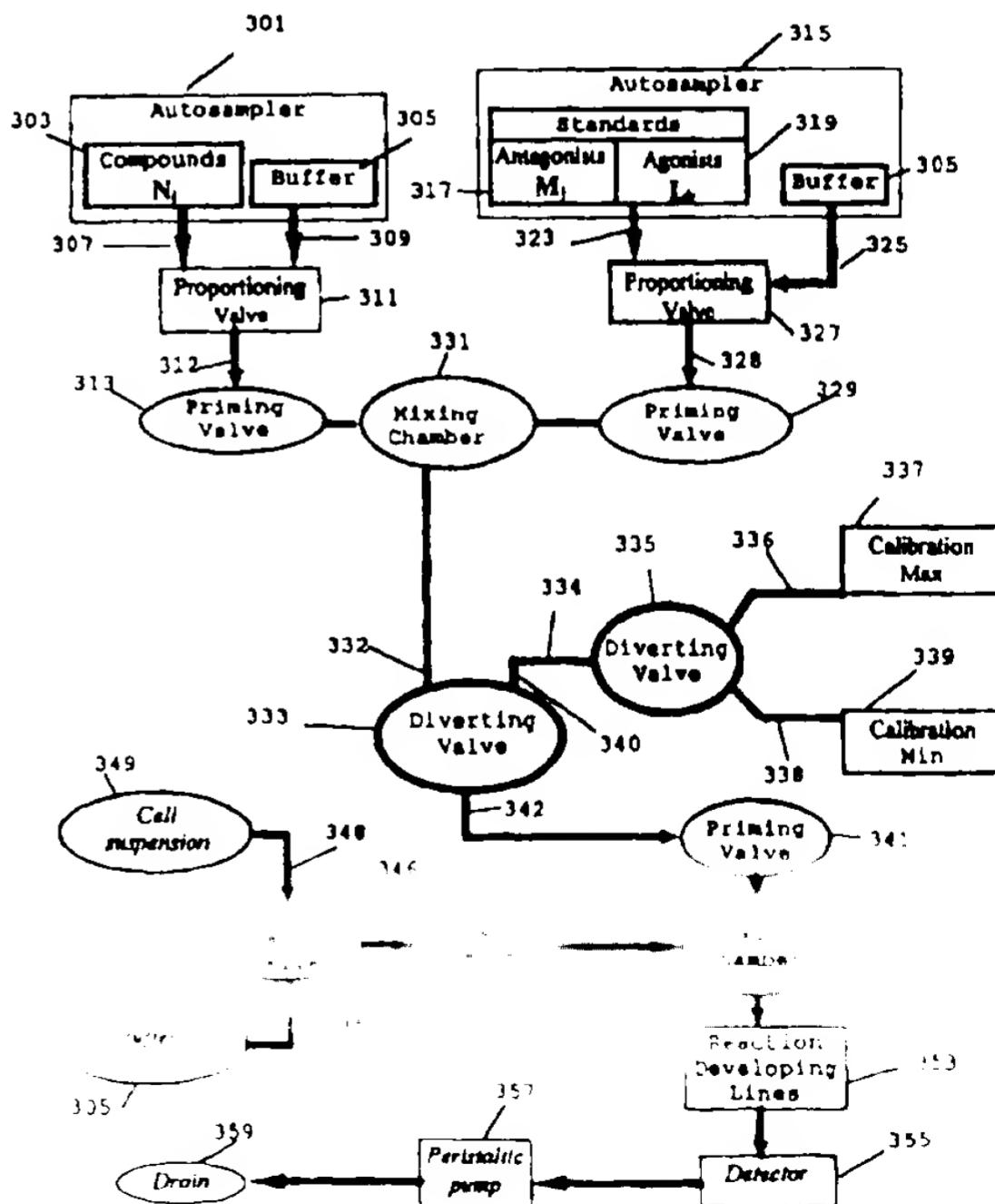
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(54) Title: APPARATUS AND METHOD FOR REAL-TIME MEASUREMENT OF CELLULAR RESPONSE

(57) Abstract

An apparatus and method for real-time measurement of a cellular response of a test compound or series of test compounds (303) on a flowing suspension of cells (349), in which a homogeneous suspension of each member of a series of cell types (349) is combined with a concentration of a test compound (303), directed through a detection zone (355), and a cellular response of the living cells is measured in real time as the cells in the test mixture are flowing through the detection zone (355). The apparatus may be used in automated screening of libraries of compounds, and is capable of real-time variation of concentrations of test and standard compounds and generation of dose/response profiles within a short timespan.



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APPARATUS AND METHOD FOR REAL-TIME MEASUREMENT OF CELLULAR RESPONSE**FIELD OF THE INVENTION**

This invention relates generally to an apparatus for screening and the pharmacological profiling of compounds modulating a cellular physiological response. This invention also relates to devices for rapid assessment of the properties of compounds that modulate the activities of cell surface receptors and ion channels. More specifically, this invention relates to methods and apparatus for detecting, evaluating and characterizing the ability and potency of substances to act as agonists or antagonists against receptors and ion channels localized on a cell surface membrane.

10

BACKGROUND INFORMATION

Biological cells contain receptor molecules located on their external membrane. The function of these receptors is to "sense" the cell environment and supply the cell with an input signal about any changes in the environment. In eukaryotic organisms such cell environment is comprised of the neighboring cells and the function 15 of the receptor is to allow cells to communicate with each other directly (the paracrine regulatory system) or indirectly (the endocrine regulatory system) thus achieving harmonized response of a tissue, organ or a whole organism. In prokaryotic cells, the surface localized receptors provide a means for detecting extracellular environment.

Having received such a signal, neurotransmitters, hormones, chemoattractant or chemorepellent substances 20 for example, the surface localized receptors transmit this information about extracellular environment into the cell through specific intracellular pathways in such a way that the cell responds in the specific fashion to accommodate these changes. When there is an altered supply of the external signal molecules or an altered activity of the cell surface molecules, the cell response would be abnormal causing malfunctioning of a tissue or an organ.

In eucariotic cells, receptor molecules determine the selective response of the cell. Each type of receptor 25 can interact only with a specific set of ligand molecules. For example, adrenergic receptors interact with adrenaline and noradrenaline, cholinergic receptors interact with acetylcholine, serotonergic receptors interact with 5-hydroxytryptamine, dopaminergic with DOPA and so on. The cells derived from the different tissues invariably express specific sets of tissue receptors. Different types of receptors are connected to different signal transduction pathways. For example, nicotinic cholinergic receptor, upon binding acetylcholine molecule, directly activates sodium channel 30 (Claudio et al., 1987, is incorporated herein by reference). G-protein coupled receptors activate enzymes of second messenger pathways, for example, adenylate cyclase or phospholipase C with subsequent activation of cAMP or phosphoinositide (see, e.g., Rybníkář et al., 1995, is incorporated herein by reference).

Activating cascade MEK/MAPK kinase Radiosensitivity Differentiation and proliferation Marshall, 1993; and Herskowitz, 1995, are incorporated herein by reference). Cytokine receptors activate JAK/STAT cascade which in 35 turn can regulate other pathways as well as activate gene transcription (Hill & Treisman, 1995, is incorporated herein by reference).

Together with the receptors, the cell surface membrane carries ion pumps, ion transporters and ion channels. These molecular assemblies work in concert to maintain intracellular ion homeostasis. Any changes in the activity of these systems would cause a shift in the intracellular concentrations of ions and consequently to the cell metabolic response.

5 Ion pumps act to maintain transmembrane ion gradients utilizing ATP as a source of energy. The examples of the ion pumps are: Na^+/K^+ -ATPase maintaining transmembrane gradient of sodium and potassium ions, Ca^{2+} -ATPase maintaining transmembrane gradient of calcium ions and H^+ -ATPase maintaining transmembrane gradient of protons.

10 Ion transporters use the electrochemical energy of transmembrane gradients of one ion species to maintain gradients of other ion counterpart. For example, the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger uses the chemical potential of the sodium gradient directed inward to pump out calcium ions against their chemical potential.

15 Ion channels, upon activation, allow for the ions to move across the cell membrane in accordance with their electrochemical potential. There are two main types of ion channels: voltage operated and ligand-gated. Voltage operated channels are activated to the open state upon changes in transmembrane electric potential. Sodium channels in the neuronal axon or L-type calcium channels in neuromuscular junctions exemplify this kind of channel. Ligand-gated channels are activated to the open state upon binding a certain ligand with the chemoreceptor part of their molecules. The classical example of ligand-gated channels is nicotinic cholinergic receptor which, at the same time, is the sodium channel.

20 There are numerous methods for detecting ligand/receptor interaction. The most conventional are methods where the affinity of a receptor to a substance of interest is measured in radioligand binding assays. In these assays, one measures specific binding of a reference radiolabeled ligand molecule in the presence and in the absence of different concentrations of the compound of interest. The characteristic inhibition parameter of the specific binding of the reference radiolabeled ligand with the compound of interest, IC_{50} , is taken as a measure of the affinity of the receptor to this compound (Weiland & Molinoff, 1981 and Swillens et al., 1995, are incorporated herein by reference). Recent advances in microchip sensor technology made it possible to measure direct interactions of a receptor molecule with a compound of interest in real time. This method allows for determination of both association and dissociation rate constants with subsequent calculation of the affinity parameter (F_gerstam et al., 1992, is incorporated herein by reference). While being very precise and convenient, these methods do not allow to distinguish between agonist and antagonist activity of the compound.

25 The type of biological activity of the compounds, agonist or antagonist, may be determined in the cell based assays. In the methods described in Harbold & Brust, 1995, which is incorporated herein by reference, cells

30 transfected with a gene encoding a receptor are used. These methods are inconvenient because they require agonist and antagonist information about compound. Such methods are inconvenient because they require very laborious manipulations with gene transfection procedures, are highly time consuming and use artificially modified cells. Besides, to prove that the agonistic effect of a particular compound is connected to the stimulation of a

transfected receptor, several control experiments with a positive and negative control cell lines should be performed as well.

Most closely related to the methods of this invention are the methods described in Parce et al., 1994, which is incorporated herein by reference. These prior art methods use natural cells and are based on registering the natural cell responses, such as the rate of metabolic acidification, to the biologically active compounds. The disadvantage of the prior art is low throughput speed, each measurement point taking about three minutes. Another disadvantage of the prior art is the use of cells immobilized on the internal surface of the measuring microflow chamber. This leads to the necessity of using separate silicon sensors, or cover slips, with the cells adherent to them for each concentration point of the agonist or antagonist, for the receptors that undergo desensitization upon binding to the agonist molecule. This results in high variability of the experimental results.

Ionized calcium, unlike other intracellular ion events, e.g. changes in the intracellular concentrations of protons, sodium, magnesium, or potassium, serves as the most common element in different signal transduction pathways of the cells ranging from bacteria to specialized neurons (Clapham, 1995, is incorporated herein by reference). There are two major pools which supply signal transduction pathways in the cell with the calcium ions, 15 extracellular space and the endoplasmic reticulum. There are several mechanisms to introduce small bursts of calcium into cytosol for signal transduction.

Both excitable and nonexcitable cells have on their plasma membrane predominantly two receptor classes, G-protein coupled serpentine receptors (GPCSR) and the receptor tyrosine kinases (RTK), that control calcium entry into cell cytoplasm. Both GPCSR and RTK receptors activate phospholipase C to convert phosphatidylinositol into inositol(1,4,5)-trisphosphate (InsP_3) and diacylglycerol. InsP_3 acts as an intracellular second messenger and activates specialized receptor that spans the endoplasmic reticular membrane. The activation of this receptor triggers release of calcium ions from the endoplasmic reticulum (Berridge, 1993, is incorporated herein by reference). The calcium ions can also enter the cytoplasm of excitable and nonexcitable cell from extracellular environment through specialized voltage-independent Ca^{2+} selective channels triggered by specific ligands. In nonexcitable cells, hyperpolarization 20 of the plasma cell membrane also enhances entry of calcium ions through passive transmembrane diffusion along the electric potential. For example, opening of potassium channels brings the membrane potential to more negative values inside the cell, thus facilitating Ca^{2+} entry across the plasma membrane. Excitable cells contain voltage-dependent Ca^{2+} channels on their plasma membrane, which, upon membrane depolarization, open for a short period of time and allow inflow of Ca^{2+} from external media into cytoplasm. The endoplasmic reticulum membrane as well as plasma 25 membrane of the excitable cells contains InsP_3 receptors and Ca^{2+} -sensitive ryanodine receptors (RyR) releasing Ca^{2+} from intracellular stores upon membrane receptor triggered phospholipase C activation or depolarization-induced short burst of Ca^{2+} entry into cell cytoplasm.

It is established that G-protein coupled serpentine receptor in the cell membrane triggers calcium mobilization through the activation of phospholipase C_β (Sternweis and Smrcka, 1992, is incorporated herein by reference) whereas tyrosine kinase receptors activate phospholipase $C\gamma$ with subsequent intracellular Ca^{2+} mobilization (Berridge & Irvine, 1989, 30 is incorporated herein by reference).

There are many plasma membrane G-protein coupled serpentine receptors, tyrosine kinase growth factor receptors and voltage- and ligand-regulated channels known to initiate intracellular Ca^{2+} mobilization.

Ca^{2+} plays an essential role in many functional processes of a cell. For example, Ca^{2+} affects the cell cycle (Means, 1994, is incorporated herein by reference) and activates specific transcription factors (Sheng et al., 1991, is incorporated herein by reference). Scores of receptors and ion channels use the Ca^{2+} signal to initiate events as basic as cell motility, contraction, secretion, division etc..

Increases in cytosolic and, consequently, in nuclear concentration of the Ca^{2+} can also be a cell death promoting signal. For example, prolonged increase in free Ca^{2+} activates degradation processes in programmed cell death, apoptosis, activates nucleases that cleave DNA and degrade cell chromatin, promotes DNA digestion by direct stimulation of endonucleases, or indirectly by activation of Ca^{2+} -dependent proteases, phosphatases and phospholipases, resulting in a loss of chromatin structural integrity (Nicotera et al., 1994, is incorporated herein by reference).

A development of intracellular fluorescent calcium indicators (Grynkiewicz et al., 1985, is incorporated herein by reference) made it possible for intracellular concentration of free calcium to be measured directly in the living cell. Thus the ability to register changes in intracellular calcium concentration provide the means for monitoring effects of different compounds useful in treating various diseases, whose action is thought to be a result of an interaction with membrane receptors and ion channels.

With the advent of combinatorial chemistry approaches to identify pharmacologically useful compounds, it is increasingly evident that there is a need for methods and apparatuses capable of performing automated characterization of pharmacological profiles and corresponding potencies of the compounds in synthesized combinatorial libraries. This would enable the rapid screening of a large number of compounds in the combinatorial library the identification of those compounds which have biological activity, and the characterization of those compounds in terms of potency, affinity and selectivity.

It is an object of this invention to provide methods for screening and the quantitative characterization of potentially pharmacologically effective compounds that specifically interact with and modulate the activity of cell membrane receptors, ion pumps and ion channels using living cells.

It is an additional object of this invention to provide methods capable of characterizing an affinity of the active compounds to the binding sites of the cell.

It is another additional object of this invention to provide methods to distinguish between agonistic and antagonistic activity of the compounds.

It is yet another additional object of this invention to provide methods to determine the nature of the receptor/ion channel at the site of action.

It is yet another additional object of this invention to provide methods to characterize cell receptor pattern for particular cell source tissue.

It is yet another additional object of this invention to perform each of the above methods on each member of a series of cell types.

It is yet another additional object of the invention to determine the pattern of cell surface receptors expressed in one or more cell types.

5 It is yet another additional object of the invention to confirm that a test compound influences the activity of a particular receptor.

It is yet an additional object of the invention to determine the activity of a given receptor in a variety of cell types in which it is expressed.

It is a specific object of this invention to provide an apparatus for fulfillment of the objectives above.

10 It is yet another additional object of this invention to provide an apparatus for fulfillment of each of the objectives above for each member of a series of cell types.

At least some of these and other objectives are addressed by the various embodiments of the invention disclosed herein.

SUMMARY OF THE INVENTION

15 The present invention addresses the above and other needs by providing a method and corresponding apparatuses which allows the automated characterization of pharmacological profiles and corresponding potencies of compounds in synthesized combinatorial libraries. This enables the rapid screening of a large number of compounds in the combinatorial library, the identification of those compounds which have biological activity, and the characterization of those compounds in terms of potency, affinity and selectivity.

20 A variety of effects caused by the compounds to be screened may be detected and quantitatively characterized according to the present invention. Preferably, these effects include but are not limited to changes in intracellular concentration of ionized calcium, cAMP or pH, transmembrane potential and other physiological and biochemical characteristics of living cell which can be measured by a variety of conventional means, for example using specific fluorescent, luminescent or color developing dyes.

25 The present invention also includes methods of screening for agonist or antagonist activity of drugs, methods of characterizing their potency profiles, methods of identifying the receptor expression pattern of cell membrane ("receptor fingerprinting") and methods of determining toxicity profiles for the compounds. In these methods, a steady flow of cells is mixed with flows of the compound and a standard substance. The effects of the compound alone and in mixture with the standard substance are measured and provide the means for pharmacological profiling of the compounds, drug screening and cell receptor pattern characterizing.

In a preferred embodiment of the invention, the compounds to be screened and standard agonist and antagonist substances are organized in a 3D architecture.

Another preferred embodiment of the invention, the non-coherent cells are grown in a suspension of freely flowing cells by growing them in an appropriate cell cultivating system.

In another preferred embodiment of the invention, the naturally adherent cells which need attachment to a surface for their growth, are grown in the appropriate cell cultivating system containing commercially available micro spherical beads to which the cells adhere during the growth.

5 In yet another preferred embodiment of the invention, the naturally adherent cells which need attachment to a surface for their growth, are grown in the cell culture flasks with a subsequent detachment of the cells from the flask bottom with an appropriate detaching reagent.

10 In accordance with the present invention, either eukariotic or prokaryotic cells can be used. The cells can be transfected with a gene coding to express a receptor of interest, for example, an orphaned receptor. In addition, the variety of compounds having biologically relevant activity may be used including but not limited to neurotransmitters, hormones, toxins, receptor activators and inhibitors, ion channels and ion pump modulators, irritants 15 and/or drugs.

15 The cells grown in accordance with the preferred embodiments described above, are mixed with an appropriate fluorescent dye, for example FURA-2AM for measurements of concentrations of intracellular calcium or BCECF-AM for measurements of intracellular pH, and are incubated in the appropriate conditions to allow the dye to penetrate into the cell. The cells loaded with a dye are supplied to the apparatus. In the apparatus, the cells are successively mixed with a solutions of the compounds to be tested.

One aspect of the present invention is a method for identifying compounds having biological activity, comprising the steps of: (a) combining a homogeneous suspension of living cells with a test compound having an unknown cellular effect to form a test mixture, (b) directing the test mixture through a detection zone; and (c) measuring a cellular response of the suspended cells to the test compound as the test mixture is flowing through the detection zone. The method will often include the additional steps of: (d) combining a homogeneous suspension of the cells with a standard compound having a known effect on the cellular response of the cells to form a standard mixture; (e) directing the standard mixture through the detection zone; and (f) measuring the cellular response of the cells to the standard compound. In one embodiment, the standard compound and the test compound are simultaneously mixed with the cells in the combining steps, and the measuring step detects the known effect or an alteration of the known effect. The standard compound can be an agonist or antagonist of the cellular response. In one mode of operation, steps (a) and (d) are performed simultaneously; steps (b) and (e) are performed simultaneously; and steps (c) and (f) are performed simultaneously using a single suspension of the cells. In another mode of operation, steps (a), (b), and (c) are performed first, and then steps (d), (e), and (f) are performed, wherein 20 the test compound is added together with the standard compound in step (d). If the cellular response is detected in step (c) to indicate that the test compound is active to generate the response, and the standard compound is an antagonist than a decrease 25 in the cellular response is detected in step (f) indicating that the test compound is an agonist. If the cellular response is detected in step (c) to indicate that the test compound is active to generate the response, and the standard compound is an agonist than an increase in the cellular response is detected in step (f) indicating that the test compound is an antagonist.

30 DETECTED IN STEP (C) INDICATING THAT THE TEST COMPOUND IS ACTIVE TO GENERATE THE RESPONSE, AND THE STANDARD COMPOUND IS AN AGONIST, THEN AN ALTERATION OF THE KNOWN EFFECT DETECTED IN STEP (F) IS INDICATIVE THAT THE TEST COMPOUND IS AN ANTAGONIST OF THE KNOWN EFFECT. PREFERABLY, THE METHOD IS PERFORMED AUTOMATICALLY UNDER THE DIRECTION OF A PROGRAMMABLE COMPUTER ON A PLURALITY OF TEST COMPOUNDS.

and a plurality of standard compounds, and a successive series of antagonists are automatically added as the standard compound in step (d) if the cellular response is detected in step (c) to indicate that the test compound is active to generate the cellular response, whereby a decrease in the cellular response detected in step (f) is indicative that the test compound is an agonist of the known effect; and a series of agonists are automatically added as the standard compound in step (d) when the cellular response is not detected in step (c), whereby an alteration of the known effect detected in step (f) is indicative that the test compound is an antagonist of the known effect.

In one embodiment of the method, if step (f) indicates that the compound is an agonist of the known effect, then the method includes automatically determining the concentration dependence of agonist activity of the test compound by repeating steps (a), (b), and (c), and (d), (e), and (f) while varying the concentration of the test compound and the standard compound and recording resultant changes in the cellular response; and if step (f) indicates that the compound is an antagonist, then the method includes automatically determining the concentration dependence of inhibition of the cellular response in the presence of the agonistic standard compound by repeating steps (d), (e), and (f) while varying the concentration of the test compound and the standard compound and recording resultant changes in the cellular response. Optionally, the method also comprises the step, when step (f) indicates that the compound is an antagonist, of: (g) automatically determining the concentration dependence of cell response activation by repeating steps (d), (e), and (f) for a zero concentration of the test compound while varying the concentration of the standard compound and recording resultant changes in cellular response, and then repeating this step (g) for different concentrations of the test compound. The method may further include the step when step (f) indicates that the compound is an agonist, of: (h) automatically determining the concentration dependence of cell response activation by repeating steps (d), (e), and (f) for a zero concentration of the standard compound while varying the concentration of the test compound and recording resultant changes in cellular response, and then repeating this step (h) for different concentrations of the compound. Variation of the concentration of the test compound and/or the standard compound can be done continuously or in a stepwise manner. One preferred step includes graphically displaying the recorded changes in the cellular response.

The cellular response can be any desired cellular response susceptible of being measured or detected as the cells flow past a detector in suspension. It can be evidenced by analyzing the cells themselves or the medium in which the cells are suspended. Cellular responses can be measured, for example, from a change in intracellular ion concentration, such as calcium, magnesium, proton, sodium, or potassium. In one embodiment, the ion is detected using an intracellular dye such as a visible and/or fluorescent dye.

In another embodiment, a method comprises the steps of: (a) combining a homogeneous suspension of living cells with a test compound having an unknown cellular effect to form a test mixture, (b) directing the test mixture through a detection zone, (c) measuring a cellular response,

(d) moving the detection zone, and repeating steps (b) through (d) for each of said series of cell types to be tested so as to measure the effect of said test compound on each cell type of said series of cell types. In this embodiment, the method may further comprise the steps of: combining a homogenous suspension of each of said cell types in said series of cell types with a standard compound having a known effect on said cellular response, and

said cells to form a series of standard mixtures; directing said series of standard mixtures through the detection zone; and measuring the cellular response of each of said cell types to said standard compound. The standard compound may be an agonist or antagonist of said cellular response.

The invention also includes an apparatus for automatically measuring the effect of a plurality of test compounds on living cells, comprising: a test compound sampler for sequentially providing samples of multiple test compounds, a cell suspension input for providing a homogeneous suspension of living cells, a mixing zone, coupled to the test compound sampler, for receiving the samples of the test compounds from the test compound sampler, and receiving the suspension of living cells from the cell suspension input and mixing each test compound with the suspension of living cells; and a detector, coupled to the mixing zone, for measuring a cellular response of the suspended cells to each test compound. The apparatus may additionally include a standard compound sampler, coupled to the mixing zone, for providing a sample of a standard compound having a known effect on the cellular response of the suspended cells, wherein the mixing zone receives the sample of the standard compound from the standard compound sampler and mixes the standard compound with the suspended cells and the detector measures the cellular response of the suspended cells to the standard compound. In one embodiment, the mixing zone simultaneously mixes the test compound and the standard compound with the suspended cells and the detector detects the known effect or an alteration of the known effect. The apparatus may also include a first gradient device, coupled to the test compound sampler, for automatically adjusting the concentration level of the test compound transferred to the mixing zone from the test compound sampler; and a second gradient device, coupled to the standard compound sampler, for automatically adjusting the concentration level of the standard compound transferred to the mixing zone from the standard compound sampler. The apparatus may further include a switching valve, coupled to the first and second gradient devices at an input of the switching valve and coupled to the mixing zone at an output of the switching valve, for selectively switching the flow of a concentration of the test compound or a concentration of the standard compound or both to the mixing zone where the test compound and/or the standard compound is then mixed with the suspension of cells. In addition, the apparatus may include a calibration unit, coupled to the switching valve, wherein the switching valve also selectively switches the flow of a calibration solution provided by the calibration unit into the mixing zone where the calibration solution is mixed with the suspension of cells.

The reaction time of the cells with the various test and standard compounds may be controlled through use of various lengths of reaction developing lines coupled to the output of the mixing zone, for receiving a mixture of the cell suspension mixed with either the test compound, the standard compound or the calibration solution, and providing a flow path for the mixture such that there is adequate time for the suspension cells to react with the test compound.

Applied to the input of the detector which may be located outside of the reaction developing lines. In one preferred embodiment the detector detects changes in intracellular ion concentration. Preferred ions are described above.

The apparatus may additionally include a controller, coupled to the first and second gradient devices, the test compound sampler, the standard compound sampler and the switching valve, for controlling their operation, and

a computer, coupled to the controller, for sending command signals to the controller in accordance with a software program implemented by the computer, wherein the computer is also coupled to the detector in order to send and receive cell response measurement signals to and from the detector. In order to automate the apparatus further, the test compound sampler can be an automated robotic sampler capable of selecting a specified test compound from a library of test compounds. A controller can be coupled to the test compound sampler, for controlling the operation of the test compound sampler, using the computer, coupled to the controller, for sending command signals to the controller in accordance with a software program implemented by the computer, thereby controlling the selection and retrieval of test compounds by the test compound sampler from the test compound library.

The apparatus can direct flow through the various elements under either positive or negative pressure. Thus, one embodiment includes a gradient pump having an input and an output, coupled to the test compound sampler, for adjusting the concentration level of the test compound transferred to the mixing zone from the test compound sampler, wherein the test compound sampler comprises a first intake nozzle for receiving the specified test compound, a second intake nozzle for receiving a buffer solution; and wherein the gradient pump is coupled to the first and second intake nozzles and receives specified concentrations of the test compound by adjusting the amount of test compound and buffer solution received by the first and second intake nozzles, respectively, wherein the buffer solution is a diluting agent of the test compound. This embodiment may also include a standard compound sampler for providing a sample of a standard compound to the mixing zone. The standard compound sampler is preferably an automated robotic sampler capable of selecting a specified standard compound from a library of standard compounds. The apparatus may also include a second gradient pump having an input and an output, coupled to the standard compound sampler, for adjusting the concentration level of the standard compound provided to the mixing zone from the standard compound sampler, wherein the standard compound sampler comprises a third intake nozzle for receiving the specified standard compound, a fourth intake nozzle for receiving a buffer solution; and wherein the second gradient pump is coupled to the third and fourth intake nozzles and receives specified concentrations of the standard compound by adjusting the amount of standard compound and buffer solution received by the third and fourth intake nozzles, respectively, wherein the buffer solution is a diluting agent of the standard compound. The apparatus may further comprise a second mixing zone coupled to the outputs of the first and second gradient pumps, for receiving and mixing the specified concentrations of the specified test compound and the specified standard compound, such that the output of the second mixing zone is provided to the first mixing zone. Another element that can be included is a calibration unit for providing a calibration solution; and a switching valve, having a first input coupled to the second mixing zone, a second input coupled to the calibration unit, and an output coupled to the first mixing zone, for switching between the flow of either a compound mixture from the second mixing zone or the calibration unit to the first mixing zone.

Mixed with the cell suspension, interacting with the apparatus can be comprised a calibration maximum solution which provides for maximal cell response when mixed with the cell suspension, a calibration minimum solution which provides for minimal cell response when mixed with the cell suspension, a diverting valve having a first input coupled to the calibration maximum solution and a second input coupled to the calibration minimum solution for switching

between the flow of either the calibration maximum solution or calibration minimum solution; and a pump, coupled to the output of the diverting valve and an input of the switching valve, for pumping either the calibration maximum or calibration minimum solution from the diverting valve into the switching valve. The positive pressure version of the apparatus can also include a second pump, coupled to an input of the first mixing zone, for pumping the suspension of cells from the cell suspension input into the first mixing zone. Reaction developing lines, having an input coupled to an output of the first mixing zone and an output coupled to an input of the detector, for providing a flow path and a reaction time delay for a mixture received from the first mixing zone and for providing the mixture to the detector, can also be included.

In a preferred embodiment, as above, the apparatus can include a controller, coupled to the first and second gradient pumps, the test compound sampler, the standard compound sampler and the switching valve, the first and second mixing zones, the first and second pumps and the diverting valve for controlling their operation; and a computer, coupled to the controller, for sending command signals to the controller in accordance with a software program implemented by the computer, wherein the computer is also coupled to the detector in order to send and receive cell response measurement signals to and from the detector.

The apparatus of the present invention can also be run under negative pressure by utilizing a pump, coupled to the output of the detector, for providing negative pressure to the apparatus, a proportionating valve, coupled to the test compound sampler, for adjusting the concentration level of the test compound transferred to the mixing zone from the test compound sampler, wherein the test compound sampler further comprises a first intake nozzle for receiving the specified test compound, a second intake nozzle for receiving a buffer solution; and the proportionating valve receives specified concentrations of the test compound by adjusting the amount of test compound and buffer solution received by the first and second intake nozzles, respectively, wherein the buffer solution is a diluting agent of the test compound. The negative pressure apparatus can also include an automated standard compound sampler capable of selecting a specified standard compound from a library of standard compounds, the standard compound sampler including a third intake nozzle for receiving the specified standard compound and a fourth intake nozzle for receiving a buffer solution; and a second proportionating valve, coupled to the third and fourth intake nozzles, for receiving specified concentrations of the standard compound by adjusting the amount of standard compound and buffer solution received by the third and fourth intake nozzles, respectively, wherein the buffer solution is a diluting agent of the standard compound. In one version, the apparatus includes a first priming valve, coupled to the output of the first proportionating valve, for receiving the specified concentration of the test compound and providing the test compound to the mixing zone, and a second priming valve, coupled to the output of the second proportionating valve, for receiving the specified concentration of the standard compound and providing the standard compound to the mixing zone.

Another aspect of the apparatus may include a third mixing zone, having an input coupled to an output of the first mixing zone and an output coupled to an input of a detector, for providing a flow path and a reaction time delay for a mixture received from the first mixing zone and for providing the mixture to the detector. The apparatus may also include a third diverting valve, having a first input coupled to the cell suspension reservoir and a second input coupled to the buffer reservoir, for adjusting the concentration of the cell suspension, wherein the buffer is a diluting agent of the cell suspension, and a fourth priming valve, coupled to the output of the third diverting valve, for receiving the

cell suspension mixture from the third diverting valve and providing this mixture to the second mixing zone. In one embodiment, the apparatus further comprises a plurality of cell suspension reservoirs.

Another aspect of the present invention is a method of characterizing the receptors present in a cell comprising the steps of (a) combining a suspension of cells with a test agent known to influence the activity of a particular receptor to form a test mixture; (b) directing said test mixture through a detection zone; (c) measuring a cellular response of said suspension of living cells to said test agent as said test mixture is flowing through said detection zone wherein a response to a test agent indicates that said cell expresses a receptor known to respond to said test agent; and (d) repeating steps (a)-(c) with a series of test agents until the effects of each test agent has been measured. The test agent may comprise an agonist, an antagonist, or a mixture of an antagonist and an agonist. In one embodiment, this method further comprises repeating steps (a)-(d) on a series of different cell types to determine the receptors expressed by each cell type.

Another aspect of the invention is a method of confirming that a test compound has an effect on the activity of a receptor comprising the steps of contacting a negative control cell type which lacks said receptor with said test agent to form a negative control mixture; directing said negative control mixture through a detection zone; measuring the cellular response of said suspension of living cells to said test compound as said negative control mixture is flowing through said detection zone; contacting cells of the same cell type as the negative control which have been engineered or induced to express said receptor with said test agent to form a test mixture; directing said test mixture through a detection zone; measuring the cellular response of the cells in the test mixture to said test agent, whereby a difference in the measured response of said cells in said test mixture relative to the measured response of said negative control cells indicates that said test agent has an effect on the activity of said receptor. The test agent may comprise an agonist, an antagonist, or a mixture of an agonist and an antagonist.

Another aspect of the present invention is a method of determining the activity of one or more receptors in a series of cell types comprising (a) combining a suspension of living cells comprising a member of said series of cell types with an agent known to influence the activity of a particular receptor to form a test mixture; (b) directing the test mixture through a detection zone; (c) measuring the cellular response of said suspension of living cells to said test agent; (d) repeating steps (a)-(c) on each member of the series of cell types until the effect of said test agent has been measured in each cell type of said series. The test agent may comprise a known receptor agonist, a known receptor antagonist a mixture of a known agonist and a known antagonist, a compound whose activity is unknown, or a mixture of a compound whose activity is unknown and a compound which is a known agonist or a known antagonist.

Certain preferred embodiments of the present invention are discussed below in more detail in connection with the drawings.

Fig. 1 is a block-diagram of one embodiment of the combinatorial screening apparatus of the present invention.

Fig. 2 is a block-diagram of a positive pressure fluidic system which may be used in a combinatorial screening apparatus of the present invention.

5 Fig. 3 is a block-diagram of one embodiment of a preferred system which may be used in a combinatorial screening apparatus of the present invention.

Fig. 4 represents a simplified algorithm of a screening mode which may be utilized in a combinatorial screening apparatus of the present invention.

10 Fig. 5 represents a simplified algorithm for a potency mode which may be utilized in a combinatorial screening apparatus of the present invention.

Fig. 6 is a flow diagram of a preferred primary mode operation which may be implemented by a combinatorial screening apparatus of the present invention. Fig. 6 comprises the combination of Figs. 6a-6d

15 Fig. 7 is a flow diagram of a preferred screening mode operation which may be implemented by a combinatorial screening apparatus of the present invention. Fig. 7 comprises the combination of Figs. 7a-g.

Fig. 8 is a flow diagram of a preferred potency mode operation which may be implemented by a combinatorial screening apparatus of the present invention. Fig. 8 comprises the combination of Figs. 8a-e.

123. Fig. 9 represents the experimental results of ET-1 dose dependent Ca^{2+} mobilization in the presence of BQ-

Fig. 10 represents the semi logarithmic transformation of the data of the Fig.9.

20 Fig. 11 represents the experimental results of dose dependent inhibition of ET-1 induced Ca^{2+} mobilization in the TE-671 cells with BQ-123.

Fig. 12 represents the experimental results of ET-1 dose dependent Ca^{2+} mobilization in TE-671 cells with the subsequent inhibition with the BQ-123

25 Fig. 13 represents a simplified algorithm for cell mapping mode which may be utilized in a combinatorial screening apparatus of the present invention

Fig. 14 represents a simplified algorithm for functional characterization of orphaned receptors mode which may be utilized in a combinatorial screening apparatus of the present invention.

30 Fig. 15 represents a flow diagram of a preferred cell mapping mode operation which may be implemented by a combinatorial screening apparatus of the present invention. Fig. 15 comprises the combination of Figs. 15a-15f

Fig. 16 represents a flow diagram of a preferred orphaned receptor determination mode operation which may be implemented by a combinatorial screening apparatus of the present invention. Fig. 16 comprises the combination of Figs. 16a-16f

DESCRIPTION OF THE PREFERRED EMBODIMENTS

35 The present invention provides for real-time, continuous monitoring and detection of the physiological or pharmacological effect of a test compound on a series of cell types or on a single cell type. In its simplest

embodiment, the present invention comprises a method and apparatus for continuously contacting a single cell suspension or a series of cell suspensions, each of which contains a single cell type included in the series of cell types to be tested, with a predetermined concentration of at least one potentially active compound, preferably with predetermined concentrations of at least two active compounds. Then, intracellular changes that occur in response to contact between the cells and the active compounds are continuously measured as the suspensions containing the cell type to be tested or a member of the series of cell types to be tested and the test compounds pass a detector.

5 It is contemplated that the present invention will be of major value in high-throughput screening; e.g., in screening a large number of candidate compounds for activity against one or more cell types. It has particular value, for example, in screening synthetic or natural product libraries for active compounds.

10 In one preferred embodiment, a test compound, a standard compound, and a cell suspension containing the cell type to be tested or a member of the series of cell types to be tested are continuously mixed together and, after an incubation period, are passed by a detector that measures the concentration in the cells or in the intracellular medium of at least one analyte. In one embodiment, the concentration of the test compound and/or the standard compound is varied over time to generate dose/response curves as output from the detector.

15 It is preferred that the apparatus of the present invention is under the control of a computer or other programmable controller. The controller can continuously monitor the results of each step of the process, and can automatically alter the testing paradigm in response to those results.

20 The incubation period after mixing of the compound or compounds and the cells can advantageously be controlled by passing that mixture through a length of tubing connecting the mixing zone with the detector. The incubation period will thus be determined by the flow rate and the length of the tubing. Incubation periods can vary by several orders of magnitude, depending on the particular analyte and the resultant reaction time. For example, the incubation period could be as little as one second or a fraction of a second for rapid or short-lived physiological responses, or as long as several minutes or even hours.

25 The analyte can be any analyte that is readily detectable by detectors and/or detector/chemistry combinations. Thus, various ion or electrolyte concentrations, colorimetric changes, optical density changes, fluorescence, luminescence, pH, gas production, and the like are all readily adaptable for use in the present method and apparatus.

30 In detecting ion or electrolyte changes, calorimetric or fluorescent dyes are one particularly preferred embodiment. For example, calcium ion is detectable by such probes as Fura-2, Indo-1, Fura Red or Quin-2, sodium ions by SBFL, proton ions by BCECF, SNAFL, DM-NERF, magnesium ions by Mag-Fura-2 or Mag-Fura-5, chloride ions by SPO, SPA or MOAE. All these dyes are commercially available, for example, from Molecular Probes, Inc., Oregon.

35 APPENDIX: APPENDED EMBODIMENT FIGURES
COMPOUND SAMPLER 101 for receiving and holding a sample of a compound to be tested; a standards sampler 103, for receiving and holding a sample of a standard solution (e.g., a buffer and/or a known agonist or antagonist) to be mixed with the compound to be tested; a concentration gradient device 105 connected to compound sampler 101 for controlling the flow volume of the compound to be tested; a

concentration gradient device 107, connected to standards sampler 103, for controlling the flow volume of the standard solution; a switching valve 111 for receiving a compound/standard solution mixture or a calibration solution and for directing them to the mixing zone 112 for mixing one of these solutions with cell suspensions containing a cell type to be tested or a member of a series of cell types to be tested; at least one cell suspension reservoir 113 (and, where a series of cell types is to be examined, a plurality of cell suspension reservoirs each of which contains a cell suspension of one of the cell types in the series to be examined); a calibration unit 117, for supplying calibration solutions to the switching valve 111 so that the calibration solutions may be mixed with the cell suspension in predetermined ratios; a reaction developing lines unit 115, for receiving the cell suspensions mixed with either the compound/standard solution mixture or the calibration solution and for transferring this mixture to a detector 119; and a drain 121 for receiving and draining the mixture from detector 119 after the cell response has been detected and measured by detector 119. A controller 109 is coupled to the test compound sampler 101, the standards sampler 103, gradient devices 105 and 107 and switching valve 111. The controller controls the above devices by receiving command signals from a computer 123 which in turn generates, sends and receives signals in accordance with a software program 125. The mixing zone 112 can constitute a chamber, a tube, a series of baffles, or any other structure in which mixing can occur.

After a compound and/or standard is mixed with cells in mixing zone 112, which is readily commercially available and in the preferred embodiment is the static mixer 125-1345 (Bio Rad), this mixture is then sent through reaction developing lines 115 so that it may be mixed more thoroughly and provided with enough time for its reagents to thoroughly react with one another. The detector 119, is connected to the reaction developing lines unit 115, and receives the cell/compound/standard mixture from the reaction developing lines 115.

In a preferred embodiment, each of the samplers 101 and 103 include a sipper nozzle which may be positioned into a vial containing a respective compound or standard solution. These samplers are well-known in the art and are readily available commercial products, e.g. A/S 300 Autosampler, Catalog # 15006330, Scientific Measurement Systems, Inc., Colorado. For handling multiple samples, conventional automated or robotic equipment (not shown) may be used to supply the apparatus with the samples. Banks of different samples, arranged in 48 or 96 well plate format for example, can easily be accommodated by such automated sampling equipment.

The gradient devices 105 and 107 are also readily available commercial devices, e.g., GP40 Gradient Pump (Dionex). Depending on the mode of action, the corresponding concentration gradient devices 105 and 107 can prepare either discrete concentrations or continuous gradients of concentrations of the compound to be tested or the standard substance (agonist or antagonist) by diluting them with buffer. For example, if a continuous curve of cell response versus compound concentration in the presence of a standard substance is desired, the computer

will instruct the gradient devices 105 and 107 to provide a continuous gradient of the respective compound and standard substance. The determined discrete concentrations of standard solution are provided to switching valve 111. Alternatively, if a continuous curve of cell response versus standard solution concentration in the presence of a compound is desired, the computer 123 will instruct the controller 109 to control the gradient devices 105 and

107 such a way that continuous gradients of the respective standard solution and a predetermined constant concentration of the respective compound are provided to switching valve 111.

The calibration unit 117, connected to the switching valve 111, supplies calibration solutions needed to calibrate the output signal, MAX and MIN, of the apparatus. In a preferred embodiment, this calibration unit consists of a diverting valve which is a readily available commercial device, e.g., the SV3-2 Diverter Valve, (Bio-Rad). The diverter valve alternates supply of two calibration solutions to the switching valve 111. The switching valve 111 combines outflows from the gradient devices 105 and 107 or, alternatively, from the calibration unit 117 with a cell flow from a cell suspension reservoir 113 (or from one of a plurality of cell suspension reservoirs 113 each of which contains a cell type in a series of cell types to be examined) and directs the mixed flow into one of the reaction developing lines 115. The switching valve 111 is of a type which is well-known in the industry and in a preferred embodiment is the 3-way microvalve 4-8-900 manufactured by General Valve Corp. The cell suspension reservoir(s) 113 are also of a type which is well known in the art and in a preferred embodiment is a regular glass beaker. Cells are maintained in suspension pending introduction into the device by simple low shear stirring.

The reaction developing lines 115 are typically tubes made from a non-corrosive material, having a specified diameter, through which the above mentioned mixture of cell suspension, compound and standard solution may flow. For example, polyethylene, polypropylene, or polytetrafluoroethylene tubing can be used. Tubing to which cells and other reagents will not stick is particularly preferred. The diameter of the tubing is a matter of choice. Capillary tubing having an inner diameter of from about 0.2mm to about 2 mm is particularly advantageous, because it allows the use of very small sample sizes.

This tubing is typically set in a winding configuration so that as the mixture flows through it, the mixture is thoroughly agitated and mixed. Even if the mixture is well mixed before introduction into the tubing, a spiral or wound configuration allows long tubing lengths in a compact area. These reaction developing lines 115 are commercially available and in one embodiment may be formed by Teflon tubing.

The mixture of a cell suspension containing a cell type to be tested or a cell type included in a series of cell types to be tested and compound/standard solution, also referred to as "reaction suspension," enters the detector's flow-through optical cell or other detection zone with a time delay or incubation period determined by the length of the reaction developing line 115. Once the reaction suspension reaches detector 119, the detector 119 can measure the cell suspension response to the specified concentration of the test compound. In a preferred embodiment, the detector 119 measures a fluorescence signal from the calcium sensitive dye, FURA 2, spectral characteristics of which depend on a concentration of the intracellular ionized calcium, in order to determine the level of cell activity. In order to make this measurement, the detector 119 alternately irradiates the reaction mixture passing through the reaction developing line 115 with two wavelengths of light, 340 nm and 380 nm, respectively, and measures the fluorescence intensities registered at 540 nm upon excitation at 340 nm and 380 nm, respectively, is transmitted to the computer 123. The computer 123 calculates the concentration of the intracellular ionized calcium in accordance with the following equation which is well-known in the art:

FLUORESCENCE INTENSITIES AT 540NM = $\frac{I_{340} - I_{380}}{I_{340} + 3I_{380}}$ X 100% MEASURED FLUORESCENCE AT 540NM

FLUORESCENCE INTENSITIES REGISTERED AT 540 NM UPON EXCITATION AT 340 NM AND 380 NM, RESPECTIVELY, IS TRANSMITTED TO THE COMPUTER 123. THE COMPUTER 123 CALCULATES THE CONCENTRATION OF THE INTRACELLULAR IONIZED CALCIUM IN ACCORDANCE WITH THE FOLLOWING EQUATION WHICH IS WELL-KNOWN IN THE ART:

$$[\text{Ca}^{2+}] = K_d (R \cdot R_{\min}) / R_{\max} \cdot R (I_f / I_b)$$

where K_d is the dissociation constant of calcium/FURA 2 complex and R_{\min} and R_{\max} are the ratios obtained in the presence of the calibration solutions MIN and MAX, respectively, and I_f and I_b are the fluorescence intensities measured at 540 nm upon excitation at 380 nm in the presence of calibration solutions MIN and MAX respectively.

- 5 A computer 123, connected to the detector 119, dictates its operation. The computer 123 is also connected to or is a part of the controller 109 which controls the first and second gradient devices 105 and 107, switching valve 111 as well as test compound sampler 101 and standard sampler 103 in accordance with a software 125 implemented within the computer 123. The detector 119 is capable of measuring the particular desired signal, whatever its origin. An optical detector 119, may be a spectrophotometer, spectrofluorometer, or a luminometer, each of which have a flow-through optical cell. These devices are well-known in the art and are commercially available. For example, one embodiment of the apparatus of the present invention may use an AMINCO-Bowman Series 2 Luminescence Spectrofluorometer (Fa-256, Spectronic Instruments, Inc.). If the detector is a direct ion measuring device, it can, for example, comprise a pH sensor or an ion selective electrode. Sodium, calcium, and potassium detectors are examples of such devices. Detectors of this type are commercially available. All of these devices can be controlled by means of a computer 123 during the data acquisition process.

The apparatus of the present invention is contemplated in two different specific embodiments. A positive pressure system may be created with piston pumps or other suitable pumps supplying reagents under positive pressure. Alternatively, a negative pressure system may be created with a peristaltic pump or other suitable pump drawing reagents through the system. The negative pressure system is the simplest embodiment, because a large number of input sources can be driven by a single downstream pump.

Fig. 2 illustrates a schematic configuration of one embodiment of a positive pressure fluidic system. In a preferred embodiment, this system operates automatically under the control of a programmable controller, as will be explained in more detail below. This embodiment includes an autosampler 201 which holds one or more compounds to be tested 203 and buffer 205 which may be mixed with the compound 203 in order to provide various concentrations of the compound 203; intake nozzles, or ports, 207 and 209 for receiving a compound and buffer, respectively and delivering the same to a gradient pump 211 which controls the concentration level and flow of a test compound 203 into a mixing zone 227; an autosampler 213 which holds one or more standards (i.e., antagonists 215 and/or agonists 217) and a buffer 219 which may be mixed with the standard 215 or 217, in order to provide various concentrations of the standard; intake nozzles, or ports, 221 and 223, for receiving a standard and buffer, respectively, and providing the same to a gradient pump 225 which controls the concentration level and flow of a standard solution (agonist or antagonist) into the mixing zone 227. Each of the gradient pumps 211 and 225, respectively,

is connected to each other through the mixing zone 227 and to a programmable controller 109 of FIG. 1 which is not shown in Fig. 2. The programmable controller 109 controls the operation of autosamplers 201 and 213 and gradient pumps 211 and 225 by supplying remote signals to start/stop the pumping. In a preferred embodiment, gradient pumps 211 and 225 may be the GP 40 Gradient Pump manufactured by Dionex.

The mixing zone 227 receives the compound solution flowing through gradient pump 211 and, optionally, a standard solution flowing through the gradient pump 225, and mixes the compound and standard substances together. A diverting valve 235 alternates the supply of either the calibration solution for *maximal* response 233 or the calibration solution for *minimal* response 237. The diverting valve 235 preferably includes two inlet tubes, one tube for each of the two different calibration solutions 233, 237, and one outlet tube connected to a pump 231. The diverting valve 235 is well-known in the art and can be implemented by the SV-3 Diverter Valve (BioRad). The controller (109 of Fig.1) which is not shown in Fig.2, sends signals to the diverter valve 235 which switches intake ports to connect one calibration solution 233 or another, 237, to the intake of the pump 231. The pump 231 for supplying calibration solutions to the fluidic system is also shown in Fig. 2. The pump 231 receives either the calibration max. solution 233 or the calibration min. solution 237 from diverting valve 235 and then pumps the received calibration solution to switching valve 229. The outlet tube of pump 231 is connected to an input of the switching valve 229. The pump 231 is advantageously a standard piston pump which is well-known in the art. In a preferred embodiment, the pump 231 is the series 1350 Soft-Start Pump (BioRad). The switching valve 229 alternates the supply of either the compound/standard mixture or one of the calibration solutions to a mixing zone 15 239. A pump 241 supplies cells from a cell suspension reservoir 243 containing a cell type to be tested (or from one of a plurality of cell suspension reservoirs 243 each containing a cell type included in a series of cell types to be examined) to the mixing zone 239. The pump 241 receives cells from the cell suspension reservoir 243 from an inlet tube and pumps the received cells through an outlet tube to one intake of the mixing zone 239. The compound/standard solutions or calibration solutions come through another intake of mixing zone 239. Both mixing 20 zones 227 and 239, are well known in the art and in a preferred embodiment may be implemented by the Static Mixer, 125-1345 (BioRad). The pump 241 can be a standard piston pump which is well-known in the art. In a preferred embodiment, the pump 241 is the series 1350 Soft-Start Pump (BioRad). The mixture of cells and compound/standard solution or cells and a calibration solution is then fed from mixing zone 239 to reaction developing 25 lines 245. The length of these lines, combined with the flow rate, determines the incubation period; i.e., the time elapsed from the point where the cells are mixed with the compound/standard mixture or the calibration mixture to the point of reaching detector 247. The detector 247 then measures the amount of cell response due to the compound/standard mixture or the calibration solution. After the cell response has been measured by detector 247, the mixture is then drained from the detector via drain 249.

Fig. 3 illustrates a schematic configuration of a preferred embodiment of the negative pressure fluidic system. As shown in Fig. 3, one preferred embodiment includes an autosampler 301 which holds one or more compounds (N_j) 303 and a buffer 305, the autosampler 301 further including two intake nozzles or ports 307 and 309 for receiving the compound (N_j) and the buffer 305, respectively; a proportionating valve 313 for determining or specifying proportions and delivering this mixture to a priming valve 315; an autosampler 315 which holds standard antagonists (M_j) 317 agonists (L_k) 319 and the buffer 305, the autosampler 315 further including two intake nozzles 323 and 325 for receiving the standard and the buffer, respectively; a proportionating valve 327 for preparing dilutions of the standard substance 317 at 35

319 with buffer 305 in predetermined or specified proportions and delivering this mixture to a priming valve 329. The proportionating valves 311 and 327 may be of any type which is well-known in the art. In a preferred embodiment these valves 311 and 327 are the series 4 miniature solenoid type valves (4-8-900 General Valve Corp.) working in a proportioning frequency mode. Similarly, priming valves 313 and 329 may be of any "normally opened" 5 type which is well-known in the art and, in a preferred embodiment, are the series 4 miniature solenoid type valves (4-39-900) manufactured by General Valve Corp.

A mixing zone 331 mixes a compound solution received from priming valve 313 and a standard solution received from priming valve 329. The first mixing zone 331, as with the other mixing zones discussed herein, can constitute a simple "Y" connector; a chamber having a diameter several times that of the tubing; a length of tubing; 10 a serpentine, baffled chamber; a chamber containing a mechanical rotating mixer; or any other suitable structure. Typically, the method will involve very small quantities of liquid (e.g., a full concentration gradient run can be accomplished with as little as 0.3 ml of sample). Thus, the volumes to be mixed are small and the mixing zone should have small internal volume and high mixing efficacy. These types of mixers are well known in the art and 15 in a preferred embodiment may be implemented by the Visco Jet® Micro-Mixer (#TCMA0120113T) manufactured by The Lee Company.

A diverting valve 335 alternates the supply of either a calibration solution for *maximal* response 337 or a calibration solution for *minimal* response 339. The diverting valve 335 includes two inlet tubes for receiving the two different calibration solutions and one outlet tube connected to another diverting valve 333. The diverting valve 333 can have two inlet tubes, one for receiving a compound/standard solution mixture and the other for receiving one 20 of the calibration solutions 337 or 339. The diverting valve 333 then alternates the supply of either the compound/standard mixture or one of the calibration solutions through an outlet tube to a priming valve 341 which then delivers the received fluid to a mixing zone 343. A third diverting valve 347 alternates the supply of either cells from a cell suspension reservoir 349 containing a cell type to be tested (or from one of a plurality of cell suspension 25 reservoirs each containing a cell type included in a series of cell types to be tested) or the buffer solution 305 through an outlet tubing 346 to a priming valve 345 which then delivers either the cells or buffer to the mixing zone 343 where it is mixed with either a compound/standard mixture or one of the calibration solutions received from priming valve 341. The diverting valves 333, 335 and 347 may be of any type which is well known in the art, for example, a SV-3 Diverter Valve (BioRad). This mixture is then sent to reaction developing lines 353 which determine 30 the time elapsed from the point where the cells are mixed with the compound/standard mixture or one of the calibration solutions to the point where the mixture reaches the detector optical cell 355. As explained above, the reaction developing lines 353 and the detector 355 may be of any type which is well known in the art.

PRESSURE REQUIREMENT: Making use of the tubing and mixtures described above flow through the various valves (311, 33, 327, 329, 333, 335, 341, 345 and 347) mixing zones (331 and 343), the reaction developing lines 353, the 35 detector 355, and finally to the drain 359. Suitable peristaltic pumps 357 are well-known in the industry and in a preferred embodiment may be implemented by a EP 1 Econo Pump (BioRad). The pumps, valves, detectors and

other active components of the system are preferably under the automated control of a programmable controller, as explained in more detail below.

The apparatus in the present invention preferably has two primary modes of action, a screening mode and a potency mode.

5 Fig. 4 shows an algorithm that may be used in the invention to detect cell response during the screening mode. First, a cell/compound mixture is provided to a detector (step 401). Next, the apparatus determines if the compound, upon contact with the cells, triggers any cell response (step 403). There are two possibilities: either the compound does not produce any response (NO), or it induces the cell response (YES). Cell response is determined by monitoring the signal from the detector for the particular analyte being detected. The control system first
10 calibrates to establish a baseline and a maximal response, and any signal from the detector falling between those values is considered to be a positive response.

If the compound causes no cell response, (NO), as measured by the monitoring part of the apparatus, the cells are successively and automatically brought into contact with a mixture of the compound and the standard substances from a predetermined set of agonist solutions (step 407). Each solution in the set contains one or more ingredients that initiates cell response in the absence of the test compound, e.g., through the stimulation of a known cell receptor, ion pump or ion channel molecules. Next, a determination is made as to whether a cell response normally triggered with a particular agonist is suppressed by the particular compound (step 411). The apparatus will keep repeating an admixture of different standard agonist substances with the compound until it detects that the cell response triggered with a particular standard agonist is suppressed in the presence of the compound, or until
15 all agonists available to the machine have been tested. If in step 411, it is determined that a cell response normally triggered with a particular agonist is suppressed by the particular compound (YES), that compound is categorized as an antagonist to the receptor R_i (step 415). After this happens, the instrument is switched over to the potency mode of action if instructed by the software managing the apparatus' performance (step 417).

If the contact of the cells with the compound does initiate the cell response, (YES), as measured by the monitoring part of the apparatus, the cells are automatically brought into contact with successive mixtures of the compound and the standard substance from a predetermined set of antagonist solutions (step 405). Each solution in the antagonist set contains one or more ingredients that block the cell response initiated by at least one known agonist through the stimulation of a known cell receptor, ion pump or ion channel molecules. Next, a determination is made as to whether a cell response triggered with the compound is suppressed in the presence of a particular standard antagonist (step 409). The apparatus will keep repeating an admixture of different standard antagonist substances with the compound until it detects that the cell response triggered with the compound is suppressed
20 in the presence of the particular standard antagonist, (YES), as measured by the monitoring part of the apparatus. If this happens, the compound is characterized as an agonist to the receptor R_i (step 413). After this happens, the instrument is switched over to the potency mode of action if instructed by the software managing the apparatus' performance (step 417).

35 The particular cell response triggered with the compound is suppressed by the particular antagonist (YES), that compound will be characterized as an agonist to the receptor R_i (step 413). After this happens, the instrument is switched over to the potency mode of action if instructed by the software managing the apparatus' performance (step 417).

By using known sets of standard agonist and antagonist substances to different receptors, it is possible to screen the compounds against several receptor types and subtypes for specificity and selectivity. When a series of cell types is to be tested, the process can be repeated for each of the cell types included in the series of cell types to be tested in order to evaluate the compounds' activities in a number of cell types.

5 For example, for the endothelin receptor, stimulation of which is indicated by an increase in intracellular concentration of ionized calcium, the following receptor subtype specific antagonists may be used: BQ-123, BQ-788, BQ-153, BQ-485, BMS-182874 PD 151, 242, and the following receptor subtype specific agonists may be used: endothelin-1, endothelin-2, and endothelin-3. Sarafotoxin S6c, IRL 1620, BQ-3020.

10 For calcium channels, there are sets of channel type specific agonists and antagonists which can be used in a preferred embodiment. For example, agonists of intracellular calcium channels are: Ins (1,4,5)P₃, Ryanodine, Caffeine, Heparin, Perchlorate, and their antagonists are: Decavanadate, Ruthenium Red and high concentrations of Ryanodine

15 In another preferred embodiment, the screening mode is used for the characterization of the cell receptor pattern, commonly known as "receptor fingerprinting". In accordance with this embodiment, a cell type to be tested or a series of cell types are screened against standard substances from a predetermined set of agonist solutions. Each solution in the set contains one or more ingredients that initiates cell response through the stimulation of a known cell receptor. In this way, the patterns of receptor expression in two or more cell types may be evaluated. When one or more of the cell types respond to the particular agonist substance, the instrument is switched over to the potency mode of action if instructed by the software managing the apparatus' performance.

20 Fig. 5 shows one algorithm that may be used in the invention in the preferred potency mode. The first step in the potency mode is to determine whether a particular compound has been categorized as an agonist or antagonist (step 501). The apparatus then prepares continuous concentration gradients of either the standard substance or the compound being tested.

25 If the compound has been categorized as an agonist for one or more of the cell types to be tested during the screening mode, the apparatus will measure and register the concentration dependence of the cell response in the responding cells (step 503). During step 503, the apparatus will generate continuous experimental activation curves for the activatory compound. From these curves, one can calculate the potency of the compound in terms, for example, of EC₅₀ (the effective concentration of an activator which causes 50% of the maximal stimulatory response of the cells)(step 505). This calculation can be performed using well known in the art curve fitting 30 software. In a preferred embodiment this software may be implemented by the graphical software PRISM, manufactured by GraphPad, Inc.

35 SCREENING MODE: The apparatus will measure and register the concentration dependence of cell response inhibition of these cell types in the presence of a standard agonist (step 507). During step 507, the apparatus will generate continuous experimental inhibition curves for the antagonist compound taken at constant concentrations of the standard agonist substance. From these curves, one can calculate the potency of the compound in terms, to

example, of IC_{50} (the effective concentration of a blocker which causes 50% of the maximal inhibitory response cells) (step 509). The calculation of the IC_{50} values can also be implemented in a preferred embodiment with the PRISM software package.

If the compound is determined to be an antagonist for one or more of the cell types to be tested, the 5 apparatus will also measure and register the concentration dependence of the cell response in these cell types to a standard agonist substance in the presence of several concentrations of the antagonist compound (step 511). During step 511, the apparatus will generate a series of continuous experimental activation curves for the standard 10 agonist substance taken at different discrete concentrations of the antagonist compound. The apparatus will then calculate the affinity and the potency of the compound in terms, for example, of pA_2 values and determine whether the antagonist is competitive or non-competitive (step 513). The pA_2 value is proportional to a negative logarithm of the binding constant of a ligand/receptor complex and is a measure of the affinity of the ligand to the receptor: the bigger pA_2 value, the higher the compound's affinity to the receptor. Practically, pA_2 value can be calculated from the shift of the activation curves in the presence of different concentrations of the antagonist compound and can be implemented by the formula:

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$$pA_2 = \text{Log}(R-1) / \text{Log } B,$$

where R is a ratio of equipotent concentrations of the standard agonist substance measured both in the presence of discrete concentration (B) of the antagonist compound and without the antagonist. In a preferred embodiment the equipotent concentrations of the standard agonist substance can be found from the PRISM software activation curves. With the above sets of experimental curves, one can evaluate the potency and the pharmacological profile 20 for the compound under investigation in accordance with Cheng-Prusoff (Cheng & Prusoff, 1973, incorporated herein by reference), Gaddum (Gaddum, 1957, incorporated herein by reference) or Schild (Arunlakshana & Schild, 1959, incorporated herein by reference) analyses.

A detailed description of the operation of the negative pressure fluidics system of Figure 3 is given below.

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The process is started when a computer prompts an operator to chose a mode of operation from a choice of three modes: system priming mode, screening mode and potency profiling mode. In the presently preferred top-level process, the first choice is to perform system priming. Referring to Figure 3, before the priming process starts, the operator is prompted to place the nozzles of intake ports 309, 325 and 351 into a reservoir 305 filled with a buffer, the nozzles of the intake ports 336 and 338 into reservoirs 337 and 339, respectively, filled with corresponding calibration solutions, and the nozzle of the intake port 348 into one of the cell suspension reservoirs 349 filled with a cell types to be tested.

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A continuation of the previous page

negative pressure fluidics system presented in the continuation of Figure 3. After entering the system priming program begins from start state 601 and enters state 602 where it sets all parameters to their initial values. These initial parameters include, but are not limited to, the internal initialization of software variables and subroutines.

Once the initial parameters are determined, autosamplers 301 and 302 (Figure 3), position their corresponding intake nozzles 307 and 323 at "zero" position (Step 604). Each autosampler, in its "zero" position, contains a reservoir 305 filled with a washing buffer. During step 604, the power supply which supplies power to proportioning valves 311 and 327 (Figure 3), to diverting valves 333, 335 and 347 (Figure 3), and to priming valves 5 313, 329, 341 and 345 (Figure 3) is turned off. In the preferred embodiment, the priming valves, 313, 329, 341 and 345, are normally opened in a non-powered state (off); the proportioning valves, 311 and 327, in the non-powered (off) state, connect their outlets 312 and 328 with respective "normally opened" intake ports, 309 and 325; and in the non-powered (off) state, the diverting valve 335 connects its common outlet 334 with the "normally 10 opened" intake port 336, diverting valve 333 connects its common outlet 342 with the "normally opened" intake port 332, and diverting valve 347 connects its common outlet 346 with the "normally opened" intake port 348.

After the system has been initialized, peristaltic pump 357 is started (Step 606) and priming valves 313 and 345 are turned on (Step 608). In the powered state, the priming valves 313 and 345 are closed. This forces the buffer flow through tubing 325, outlet tubing 328 of proportioning valve 327, priming valve 329, mixing zone 331, intake tubing 332 and outlet tubing 342 of the diverting valve 333, priming valve 341, mixing zone 343, a 15 reaction developing line 353, detector 355, pump 357 and then into a drain container 359.

Referring again to Fig.1, computer 123 implements software code 125 to provide control signals to a controller 109 which controls the various valves, chambers etc. of the system. This control signal is delayed in order to provide adequate time for liquid to fill the fluidic lines (Step 610). Next, the proportioning valve 327 is turned on (Step 612). While proportioning valve 327 is in the powered state, the buffer located at "zero" position of the 20 autosampler 315 will flow through tubing 323, outlet tubing 328 of proportioning valve 327, priming valve 329, mixing zone 331, intake tubing 332 and outlet tubing 342 of diverting valve 333, priming valve 341, mixing zone 343, a reaction developing line 353, detector 355, pump 357 and then into a drain container 359.

After energizing the proportioning valve 327, the control signal is delayed again (Step 613) in order to provide the time needed for the buffer to fill out the fluidics system. After the delay in Step 613, the control signal 25 will set the proportioning valve 327 to its off position (Step 614), turn off (open) priming valve 313 (Step 616) and turn on (close) the priming valve 329 (Step 618). This will fill out fluidic lines 309, 312, 332, 342, priming valve 341, mixing zone 343, a reaction developing line 353, detector 355 and pump 357 during a delay (Step 619) which is needed for the lines to be filled with buffer 305.

Next, the proportioning valve 311 is powered (Step 620) for a time duration controlled by Step 621. During 30 this delay period, the same fluidic lines are being fed with the buffer located at "zero" position of the autosampler 301, coming through the nozzle 307

At the end of the delay period, the proportioning valve 311 is turned off (Step 624) and turns on both diverting valve 335 and diverting valve 333 (Step 626). During a delay period, determined by Step 627, the liquid flow will be directed from intake tubing 338 and 35 outlet tubing 334 of the diverting valve 335 through the intake tubing 340 and the outlet tubing 342 of the diverting valve 333 through the priming valve 341, the mixing zone 343 and further through the reaction developing line 353.

corresponding compound as "negative" (Step 726). In this case, the computer 123 controls a set of agonists and calculates corresponding coordinates for positioning the nozzle 323 of autosampler 315 over the agonist containing reservoirs located in rack 319.

5 Each time the condition, " $SN_i > BS$ ", is satisfied, the program flow will go through loop K-0 of the incremental counter M (Step 730). The incremental counter, M, increases the count number by one each time it is triggered and thus determines the successive positions of nozzle 323 of autosampler 315 which serves the set of standard antagonists located on rack 317. Next, computer 123 checks if the numerical content of the incremental counter (M_j) exceeds the maximum number of standards to be tested (M_{max}) entered by the operator (Step 732).

10 If M_j is less than or equal to M_{max} , autosampler will position nozzle 323 into the standard antagonist reservoir, located on rack 317 corresponding to the numerical content of the incremental counter, M_j (Step 734). Next, proportioning valve 327 is switched over to connect its "normally closed" intake port 323 with the outlet port 328 (Step 736). During Step 736, the combined flow through mixing zone 343, reaction developing lines 353 and detector 355 is composed of one portion of standard antagonist coming from proportioning valve 327, one portion of the compound to be tested, coming from proportioning valve 311 and one portion of the cell suspension containing 15 a cell type to be tested coming from diverting valve 347.

20 Next, detector 355 registers and saves (Step 738) for further comparison, a signal, SN_iM_j , produced by a given compound, N_i , in the presence of a given standard antagonist, M_j . Then, both proportioning valves 311 and 327 are turned off (step 740). This closes intake ports 307 and 323 and opens intake ports 309 and 325 to the corresponding outlet ports 312 and 328. Autosampler 315 then positions nozzle 323 into "zero" position occupied by wash buffer reservoir 305. The proportioning valve 327 is then turned on again to open its "normally closed" intake port 323 into the outlet port 328 (Step 744) and the content of nozzle 323 is washed out (Step 746). Next, proportioning valve 327 is turned off to close intake port 323 and to open "normally opened" intake port 325. During this process, the fluidic lines are washed with buffer coming from nozzle 325 of autosampler 315.

25 Next, Computer 123 (Fig. 1) compares signal, SN_i , measured in the presence of a compound N_i alone, with the signal, SN_iM_j , measured in the presence of both a compound N_i and a standard antagonist, M_j (Step 750). If the signal in the presence of a standard antagonist, M_j , is lower than the signal generated by a compound N_i alone, $SN_iM_j < SN_i$, then both the compound N_i and the standard antagonist, M_j , is flagged as "positive" (Step 751). If both signals are equal to each other, then the compound N_i is flagged as "positive" and the standard antagonist M_j is flagged as "negative" (Step 752). After the data is flagged, the data, containing the ID number of the compound N_i with its flag value and ID number of the standard antagonist M_j with its flag value is transferred into a database 30 (Step 753). After the data is saved the program loops back to trigger the incremental counter, M, to increase its content by one (Step 730).

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35 Once the condition determined during Step 750 is met, that is the compound has been tested against all standard antagonists in a given set, the program resets the content of incremental counter M to zero (Step 755), and both autosampler 301 and autosampler 315 position their respective nozzles 307 and 323 to "zero" position, where the reservoirs with a washing buffer 305 are located (Step 756). Next, both proportioning valves

311 and 327 are turned on (Step 758). This opens "normally closed" intake ports 307 and 323 to the corresponding outlet ports 312 and 328. During this washing delay period determined by Step 760, the contents of both nozzle 312 and nozzle 328 are washed out with buffer 305. After Step 760 is finished, both proportioning valves 311 and 327 are turned off (Step 762). After the washing delay period (Step 760), the program loops back to the incremental counter N content of which is incremented by one (Step 714) by triggering signal S_{tr} from the computer 123 (Fig. 1). Then, the numerical content N_i of counter N is compared with the maximum number, N_{max} , of the compounds to be tested. If N_i exceeds N_{max} , the content of incremental counter N is zeroed (Step 717) and the screening mode is stopped (Step 719). Otherwise, Step 715 will be repeated with the next compound.

If the condition of the decision process 724, $SN_i > BS$, is not satisfied, the N_i compound is flagged as "negative" (Step 726) and the incremental counter L is triggered (Step 764). The incremental counter L is increased by one (Step 764) each time it is triggered and thus determines the successive positions of nozzle 323 of autosampler 315 relative to the set of standard agonists located on rack 319. Next, the numerical content, L_k , of incremental counter L is compared with the maximum number, L_{max} , of standard agonists to be used (step 766).

If L_k is less than or equal to the L_{max} , autosampler 315 positions nozzle 323 into the standard agonist reservoir in rack 319, which corresponds to the numerical content, L_k , of incremental counter L. Next, proportioning valve 327 is turned on to open its "normally closed" intake port 323 to outlet port 328 and proportioning valve 311 is turned off to close its intake port 307 and open intake port 309 to outlet port 312 (Step 770). During step 770, the combined flow coming through mixing zone 343, reaction developing lines 353 and detector 355 is composed of one portion of standard agonist 319 coming from inlet port 323 of proportioning valve 327, one portion of buffer 305, coming from inlet port 309 of proportioning valve 311 and one portion of the cell suspension containing a cell type to be tested coming from the diverting valve 347.

After a delay period provided by Step 772, which is needed for the mixing process to stabilize, a signal, SL_k , produced by a given standard agonist, L_k is registered and saved (Step 774). Next, proportioning valve 311 is turned on to open its "normally closed" intake port 307 to outlet port 312 (Step 776). A delay period needed for the mixing process to stabilize is provided in Step 778, after which a signal, $SL_k N_i$, is stimulated by a given standard agonist, L_k , in the presence of a given compound, N_i is registered and saved in (Step 780).

After the signal $SL_k N_i$ is registered (Step 780), both proportioning valves 311 and 327 are turned off to close their respective "normally closed" intake ports 307 and 323 and to open intake ports 309 and 325 to the corresponding outlet ports 312 and 328 (Step 782). Next, autosampler 315 positions the nozzle of intake port 323 to its "zero" position which corresponds to washing buffer reservoir 305 (Step 784). Then, the proportioning valve 327 is turned on (Step 785). This opens the "normally closed" intake port 323 to outlet port 328.

After the signal $SL_k N_i$ is registered (Step 780), both proportioning valves 311 and 327 are turned off to close their respective "normally closed" intake ports 307 and 323 and to open intake ports 309 and 325 to the corresponding outlet ports 312 and 328 (Step 782). Next, autosampler 315 positions the nozzle of intake port 323 to its "zero" position which corresponds to washing buffer reservoir 305 (Step 784). Then, the proportioning valve 327 is turned on (Step 785). This opens the "normally closed" intake port 323 to outlet port 328.

After the signal $SL_k N_i$ is registered (Step 780), both proportioning valves 311 and 327 are turned off to close their respective "normally closed" intake ports 307 and 323 and to open intake ports 309 and 325 to the corresponding outlet ports 312 and 328 (Step 782). Next, autosampler 315 positions the nozzle of intake port 323 to its "zero" position which corresponds to washing buffer reservoir 305 (Step 784). Then, the proportioning valve 327 is turned on (Step 785). This opens the "normally closed" intake port 323 to outlet port 328.

If $SL_k N_i$ is less than SL_k , then the corresponding standard agonist, L_k , is flagged as "positive" (Step 790). If both signals are equal to each other, then the standard agonist, L_k , is flagged as "negative" (Step 792). After the data is flagged, the data, containing the ID number of the compound N_i with its flag value and the ID numbe

of the standard agonist L_k with its flag value is transferred into a database (Step 794). After the data is saved the program triggers the incremental counter L (Step 764) to increase its count by one and Step 796 is repeated until condition 766 is achieved.

When the condition determined in Step 766 is met, that is the compound has been tested against all
5 standard agonists in a given set, the content of incremental counter L is reset to zero (Step 755) and both
autosampler 301 and autosampler 315 position their respective nozzles 307 and 323 to "zero" position where the
reservoirs with washing buffer 305 are located (Step 756). The system then turns on both proportioning valve 311
and proportioning valve 327 to open "normally closed" intake ports 307 and 323 to the corresponding outlet ports
312 and 328 respectively (Step 758). During a washing period provided by Step 780, the contents of both nozzle
10 307 and nozzle 323 are washed out with the buffer 305. Next, both proportioning valves 311 and 327 are turned
off (Step 762) and incremental counter N is incremented by one (Step 714). Next, the new triggered numerical
content N_i of the incremental counter N is compared with the maximum number, N_{max} , of compounds to be tested
(Step 716). If N_i exceeds N_{max} , the counter N is set to zero in step 717 and the screening mode is stopped (Step
15 719). Otherwise, the whole cycle will be repeated with a subsequent compound.

After the screening mode is stopped (Step 719), the operator is prompted by the computer to choose either
potency profiling mode or to repeat screening mode with a new set of compounds.

A continuous flow diagram of the presently preferred potencyprofiling mode with the negative pressure
fluidics system of Fig. 3 is shown in Figs. 8a - 8e.

The potency profiling program begins from a start state 800 and enters state 802 where it sets all
20 incremental counters, L, M, and N, to zero and sets all parameters to their initial values. This initial parameters
include, but are not limited to, the internal initialization of hardware and software variables and subroutines. Next,
the incremental counter N is started (Step 804), and its numerical content, N_i , is compared with the maximum
number, N_{max} , of standards, entered by the operator before starting the program (Step 806). If N_i exceeds N_{max} ,
the incremental counter N is zeroed (Step 808), and the program stops (Step 810). Otherwise, the program will
25 proceed by turning on pump 357 (Step 816). At this point, in accordance with initial parameters setup in Step 802,
the nozzles 307 and 323 of autosamplers 301 and 315 are in zero position, proportioning valves 311 and 327,
priming valves 313, 329, 341, and 345, and diverting valves 335, 333, and 347 are turned off. During step 816,
the combined flow coming through mixing zone 343, reaction developing lines 353 and detector 355 is composed
30 of one portion of buffer 305 coming from inlet port 309 of proportioning valve 311, one portion of buffer 305,
coming from inlet port 325 of proportioning valve 327 and one portion of the cell suspension coming from the
diverting valve 347. Next, diverting valve 333 is turned on (Step 820) opening "normally closed"

W1 through tube 340, which is connected to outlet tubing 334 of diverting valve 335, through intake port 340
35 of diverting valve 333, through priming valve 341 and then into mixing zone 343, where it is mixed with cells coming
from the cell suspension, through intake tube 348 and outlet tube 346 of diverting valve 347, and then through
priming valve 345 into the mixing zone 343. The cells calibration mix mixture will then flow to developing lines 353

and then to detector 355. Next, the maximum signal is measured and registered (Step 824). Diverting valve 335 is then turned on (Step 832) which opens the "normally closed" intake port 338 to outlet port 334 which allows a calibration min solution 339 to flow through diverting valve 333, priming valve 341 and into mixing zone 343 where it is mixed with cells coming from the cell suspension, through intake tube 348 and outlet tube 346 of diverting valve 347, and then through priming valve 345, into mixing zone 343. The cells/calibration min. mixture then flows to developing lines 353 and then to detector 335. The minimal response signal is then measured and registered (step 836). Next, both diverting valves 335 and 333 are turned off (Step 844). During step 844, the combined flow coming through mixing zone 343, reaction developing lines 353 and detector 355 is composed of one portion of buffer 305 coming from inlet port 309 of proportioning valve 311, one portion of buffer 305, coming from inlet port 325 of proportioning valve 327 and one portion of the cell suspension coming from the diverting valve 347. This is needed to wash out the remains of calibration solution from the fluidics line. Next, the "flag" value of the compound, N_i , is checked (Step 812).

If the "flag" value of the N_i compound is "positive", then the system will go to step 850, otherwise it will go to step 876. If the "flag" value of the N_i compound is "positive", autosampler 301 will position nozzle 307 into a reservoir corresponding to the N_i compound, located on rack 303 (Step 850). Next, proportioning valve 311 is operated in a gradient mode (step 852). In the gradient mode, the proportioning valve, 311, combines flows from two intake ports, 307 and 309, in such a way that the proportion of a N_i compound flow and a buffer flow in an outlet port, 312, changes in time to create a discontinuous change of the compound concentration. While the concentration is being changed in time, the fresh portions of the cells, coming into the mixing zone 313 from the cell suspension, at any given time will react with the different concentrations of the N_i compound. The resulting dose response dependence of the signal is registered in Step 854 with the subsequent calculation of the activation parameters for the N_i compound in Step 856 and then saving the data in a database (Step 858).

Next, an incremental counter of the standard antagonists, M, is started (Step 860) and the numerical content, M_j , of the counter M is compared with the maximum value, M_{max} , entered by the operator (Step 861). If the numerical value M_j does not exceed M_{max} , then the 'flag' value of the M_j standard is checked (Step 862). If the "flag" value of the M_j standard is not "positive", the incremental counter, M, is increased by one (Step 860), and the steps following Step 860 will be repeated as described above. If the "flag" value of the M_j standard is "positive", autosampler 315 positions nozzle 323 into the reservoir corresponding to the M_j standard antagonist, located on rack 317 (Step 864). After nozzle 323 is immersed into the standard solution, proportioning valve 311 is turned on (Step 866). When the proportioning valve 311 is turned on, it opens its "normally closed" intake port 307 which is immersed, in Step 850, into a reservoir corresponding to the next standard antagonist.

35 DURING STEPS 868 AND 869, THE FLOW IS DIVERTED FROM THE INLET PORTS 323 AND 325 THROUGH VALVE 347, DIVERTING VALVE 347, PRIMING VALVE 345 TO MIXING ZONE 343 AND FURTHER TO DEVELOPING LINE 353 AND DETECTOR 355. NEXT, PROPORTIONING VALVE 327 IS OPERATED IN A CONCENTRATION GRADIENT MODE (STEP 868). IN THE GRADIENT MODE, PROPORTIONING VALVE 327 COMBINES FLOWS COMING FROM THE TWO INTAKE PORTS, 323 AND 325 INTO OUTLET PORT 328 IN SUCH A WAY THAT THE PROPORTION OF A M STANDARD ANTAGONIST FLOW AND A BUFFER FLOW CHANGES IN

-29-

time to create a discontinuous change of the standard antagonist concentration. While the concentration is being changed in time, the fresh portions of the cells flow into mixing zone 343 from the cell suspension and react with the mixture composed of the N_i compound at constant concentration and the standard antagonist M_j at the concentration different at any given time. The resulting dose response inhibition by the M_j standard antagonist of the N_i compound stimulated signal is registered in Step 870, with the subsequent calculation of the inhibition parameters for the M_j standard antagonist in a Step 872 and saving the data in a database (Step 874).

After the curve is registered in Step 870 both proportioning valves 311 and 327 are closed (Step 871) and the program flow returns to Step 860 where incremental counter M is incremented by one and the program returns to Step 861 where M_j is compared to M_{max} .

When all of the standard antagonists M_j are counted and those with "positive flags" are measured, the numerical value M_j becomes greater than M_{max} and the decision process in Step 861 returns to Step 804 where the incremental counter N increases its numeric value by one. The value of N_i is then compared to N_{max} , the maximum number of compounds to be tested (Step 806). If N_i exceeds N_{max} , the incremental counter N is reset to zero (Step 808), and the program stops (Step 810). If multiple cell types are to be evaluated, this process can be repeated for each of the cell types in the series of cell types to be tested.

If, in Step 812, the flag value of the N_i compound is determined to be "negative", a incremental counter L which counts the standards, L_k , in a set of agonists, located in rack 319, is updated (Step 876). Next, the numerical value L_k is compared with the maximum number, L_{max} , of standard agonists entered by an operator (Step 877).

Until the numerical value L_k of the L counter is greater than the L_{max} value, the program will go to Step 878, where the 'flagged' value of the L_k standard is checked. If the flag value is not positive, the program returns to Step 876. If the flag value of the L_k standard is positive, autosampler 301 positions its nozzle 307 into a corresponding reservoir containing the N_i compound located in rack 303 (Step 880), autosampler 315 positions its nozzle 323 into a corresponding reservoir containing the L_k standard agonist, located in rack 319 (Step 881).

Next, the concentration gradient mode of proportioning valve 327 is activated (Step 882) and a dose response stimulation of a signal with the L_k standard agonist is then registered (step 884). In the gradient mode, proportioning valve 327 combines flows coming from the two intake ports, 323 and 325, into outlet port 328 in such a way that the proportion of a L_k standard agonist flow and a buffer flow changes in time to create a concentration gradient of the standard agonist. While the gradient is being created in time, fresh cells flow into mixing zone 343 from the cell suspension through "normally opened" intake tubing 348 and outlet port 346 diverting valve 347 "normally opened" priming valve 345 and discontinuously react with the standard agonist at the continuously changing concentration. The standard dose-response dependence (the signal stimulation) of the standard agonist is registered (Step 884), the activation parameters are calculated (Step 892) and then saved in a database (Step 894).

After the gradient mode of proportioning valve 327 is finished, the valve stays either at the proportion rate reached by the end of the gradient mode or at predetermined proportion rate which establishes the constant

concentration of the L_k standard agonist which will be used in further steps. Next, the concentration gradient mode of proportioning valve 311 is activated (Step 886). A dose response inhibition by the N_i compound of the L_k standard agonist stimulated signal is then registered (Step 888) with a subsequent calculation of the inhibition parameters in Step 896. This data is then saved in a database (Step 894). When the registration of the concentration dependence is finished both proportioning valves 311 and 327 are turned off (Step 890) to close their intake ports 307 and 323 and to open "normally opened" intake ports 309 and 325. During this process, the fluidics lines are washed with buffer 305 coming from nozzles 309 and 325 of autosamplers 301 and 315 through outlet ports 312 and 328 of proportioning valves 311 and 327, priming valves 313 and 329, mixing zone 331, "normally opened" intake port 332 and outlet port 342 of diverting valve 333, "normally opened" priming valve 341, mixing zone 343, developing line 353 and detector 355. Next, the program returns to Step 876, at which point the above cycle is repeated until all the standard agonists are counted, and those with a positive flag are measured, and the numerical value L_k of the incremental counter becomes greater than the L_{max} value, at which point, the program will return to Step 804.

When all of the compounds N_i are counted (Step 804) and tested, the N_i number becomes greater than the N_{max} value and the incremental counter N is reset to zero (Step 808) and the program stops (Step 810).

Cells for use in the apparatus can be selected for the presence of particular known receptors or for their ability to provide predetermined cellular responses to particular stimuli. A large number of such cells are known. For example, to measure the effect of compounds on calcium mobilization induced by different types of receptors, one may wish to use Jurkat T Cells, Platelets, Umbilical Vein, Endothelial Cells, or Chinese Hamster Lung Fibroblasts for thrombin receptor; Cerebellar Purkinje Cells, Cortical Astrocytes and Cortical Glial cells for AMPA receptors; Hippocampal Neurons for NMDA Receptor; P-12 cells for Purinergic Receptors; Oligodendrocytes for Platelet-Derived Growth Factor Receptor, Human Neuroblastoma cells and Pituitary Cells for Neuropeptide Y Receptors and protein-tyrosine kinase and protein-tyrosine phosphatase receptors; Human Medulloblastoma cells for Endothelin Receptor; Neutrophils for TNF α Receptor; NG108-15 cells for opioid, bradykinin and ATP; Synovial Fibroblasts for Plasminogen Receptors and so on. If one wishes to measure an intracellular ion concentration, for example, one can preincubate the cells with a dye or other detectable material having sensitivity to concentration of that particular ion. (An actual working example illustrating preparation of cells for detection of calcium ion is set forth in Example 1.)

Alternatively, if one wishes to determine the pattern of natural expression of receptors responsible for Ca^{2+} signaling pathway, then one may use the cells of particular interest and then using a set of agonists known to exert their activity through Ca^{2+} mobilization, to characterize the cells by what type of the receptors are expressed in these particular cells. This set of agonists may consist of ionophores such as

The following examples illustrate specific, non-limiting experiments, or compound profiling operations, in accordance with the present invention.

EXAMPLE 1

TE-671 cells, human medulloblastoma (ATCC CRL 8805), naturally express endothelin subtype A receptor ET_AR. This receptor belongs to the family of seven transmembrane-spanning G-protein coupled receptors and is known to activate calcium mobilization in the cell cytoplasm upon binding to its specific agonist endothelin-1, a 21-amino acid peptide. To characterize the affinity of the agonist for the receptor, conventional methods measure the physiological response of the cell in the presence of several concentrations of the agonist or antagonist (Sakamoto, A. et al., 1994, incorporated herein by reference).

Fig. 9 illustrates a measurement of the activation of intracellular calcium mobilization (Ca^{2+} measured in nanomolar concentration) as a function of the concentration of endothelin-1 (ET-1, measured in picomolar concentration), using the apparatus and method of the present invention. The apparatus was a negative pressure computer controlled unit as described above. This run was performed in the presence of several concentrations of ET_AR specific competitive antagonist BQ-123 (shown in nanomolar concentration).

TE-671 cells were prepared for use in the cell physiometer of the present invention by growing them in a T75 flask until confluence. The growth media was decanted and the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and supplemented with a fresh DPBS containing 2 μM FURA-2AM, which easily penetrates into cell cytoplasm and is hydrolyzed there with nonspecific esterases to form FURA 2, a dye which is sensitive to the ionized form of calcium. After 30 min incubation, the cells were supplemented with the equal volume of fresh DPBS and incubated for further 30 min. The cells, now loaded with the dye, were detached from the flask bottom with 0.05% Trypsin-EDTA solution. The cells were then washed twice with DPBS, containing 0.1% soybean trypsin inhibitor, and resuspended in calcium-free, magnesium-free and phenol red-free Dulbecco's modified Eagle's medium (DMEM).

Briefly, with reference to Figure 3, after calibration with minimum and maximum standards (339, 337), ET-1 solution, an agonistic test compound, was mixed with DPBS buffer in continuously changing proportion by the proportioning valve 311 and was directed through the proportioning valve 311, mixing zone 331, diverting valve 333, and priming valve 341 into the mixing zone 343, where it was mixed with a suspension of TE-671 cells to provide a final concentration of cells 4×10^5 cells/ml. At the same time, BQ-123, as a standard antagonist solution, was also directed to the mixing zone 331 at a predetermined concentration prepared by mixing BQ-123 with DPBS in the proportioning valve 327, in a ratio of 1:1 with the ET-1 solution. In mixing zone 343, the mixed flow of BQ-123 at constant concentration, and ET-1 at a continuously changing in time concentration is mixed with cells in a ration of 2:1. This gives three fold final dilution for each component of the flow passing through mixing zone 343 and eventually, detector 355. The proportioning valve 327 was programmed for the first, second and third

30 31 32 33 34 35
PROPORTIONING VALVE DIVERTING VALVE PRIMING VALVE
CHANGES IN INTRACELLULAR CALCIUM CONCENTRATION IN THE TE-671 CELLS PASSING THROUGH THE DETECTOR 315, WERE MEASURED IN REAL TIME AS A FUNCTION OF ET-1 CONCENTRATION USING FLUORESCENT DYE FURA-2 AS DESCRIBED ABOVE.

The flow rate and the length of the reaction developing line were chosen so that the time interval from the point of mixing the cells with the compound to the point of signal detection in the flow-through optical cell was 40 seconds. The time required for the complete concentration gradient run to generate the curves in Figure 9 was five minutes using the present invention. In contrast, in a conventional assay using the phosphatidylinositol turnover rate as an indicator of ET_AR stimulation, it usually takes several hours to get only a few concentration points on the response curve, and it would take an inordinate amount of time to generate a complete curve as shown in Figure 9.

Figure 10 is a logarithmic transformation of the data in Fig. 9, which is used in the art to calculate maximal cell response in the presence of antagonist as well as EC₅₀ value for agonist and pA₂ value for antagonist. The EC₅₀ value calculated from the activation curve without BQ-123 is equal to 10 pM and one can see that the activation curves in Figure 10 are parallel shifted to the right in the presence of BQ-123, which is known in the art as characteristic for competitive type of inhibition. The average pA₂ value calculated from the two different concentrations of BQ-123 is equal to 7.96. This corresponds to inhibition constant, K_i, for BQ-123 of 11 nM. The EC₅₀ and pA₂ values are in close agreement with the literature data (Masaki Ihara et al., 1992, is incorporated herein by reference).

EXAMPLE 2

Fig. 11 illustrates the inhibition of ET-1 induced intracellular calcium mobilization with BQ-123 in TE-671 cells. The same cell physiometer apparatus and cell preparation procedure was used here as in Example 1.

In this experiment, ET-1 was introduced at a constant concentration from the "standard" sipper nozzle of the inventive apparatus and BQ-123 was introduced through the "compound" nozzle of the apparatus. In this example, as in Example 1, the concentration run was five minutes and the reaction time (from mixing to detection) was forty seconds.

Briefly, with reference to Figure 3, after calibration with minimum and maximum standards (337, 339), ET-1 solution, an agonist standard solution, was directed at a predetermined concentration prepared by proportioning valve 327 by mixing it with DPBS buffer, through priming valve 329, mixing zone 331, diverting valve 333 and priming valve 341 and then into mixing zone 343, where it was mixed with a suspension of TE-671 cells to provide a final concentration of cells of 4×10^5 cells/ml. At the same time, DPB in volumetric ratio of 1:1 to ET-1 solution was directed through intake port 309 and outlet port 312 of proportioning valve 311, priming valve 313 to mixing zone 331 and further through fluidics system to the detector 355. After the intracellular calcium concentration in the presence of the standard agonist is stabilized, BQ-123, as an antagonist, is introduced at a continuously changing

concentrations prepared by mixing BQ-123 with DPBS in proportioning valve 311, through the system in a volumetric ratio of 1:1 with the ET-1 solution. In mixing zone 343, the mixed flow of ET-1 at constant concentration, and BQ-123 at a continuously changing in time concentration was mixed with cells in a ratio of 2:1. This gives three fold final dilution for each component of the flow passing through mixing zone 343 and eventually, detector 355. The

proportioning valve 327 was preprogrammed to give a final concentration of ET-1 of 40 pM at the detector. The proportioning valve 311 was preprogrammed to give final concentration of BQ-123 from 0 to 300 nM. Changes in intracellular calcium ion concentration in the TE-6712 cells passing through the detector 355, were measured in real time as a function of BQ-123 concentration using fluorescent dye FURA-2 as described above.

5

EXAMPLE 3

Fig. 12 shows the "null method" experiment design (Lazareno & Birdsall, 1993, incorporated herein by reference). This example uses the same instrument and the same reagents as in Examples 1 and 2.

In this example, a first concentration gradient of ET-1 coming from the "standard" nozzle 323 of the 10 inventive apparatus is prepared with the buffer solution coming from the "compound" nozzle. When the final ET-1 concentration has reached the value of 40 pM, the gradient device 1 keeps the ET-1 concentration constant and gradient device 2 starts raising the concentration of the BQ-123 coming from the "compound" nozzle. One can switch the agonist gradient over to an antagonist at any predetermined concentration of the agonist.

EXAMPLE 4

15 The devices of the present invention may be used to determine the pattern of cell surface receptors expressed in one or more cell types as follows. Each of the different cell types to be tested is placed in one of a plurality of cell suspension reservoirs, or, if a single cell type is to be examined, an apparatus with a single cell suspension reservoir may be used to hold the cells. The effects of one or more test agents known to influence the

The devices of the present invention may be used to confirm that a test agent influences the activity of a particular receptor as follows. The test agent may be an agonist, an antagonist, or a mixture of an agonist and antagonist. The test agent may be an agent which is known to influence the activity of the receptor or an agent whose effect on the receptor is unknown. A cell type lacking the receptor serves as a negative control and is placed in one of the cell suspension reservoirs. A cell of the same cell type as the negative control which has been engineered or induced to express the receptor is placed in another cell suspension reservoir. The effects of one or more test agents is assessed in each of these cell types by contacting the cells with the test agent to form a negative control mixture and a test mixture, directing the negative control mixture and the test mixture through a detection zone, and measuring the cellular responses of the negative control cells and the engineered or induced cells.

5 in one of the cell suspension reservoirs. A cell of the same cell type as the negative control which has been engineered or induced to express the receptor is placed in another cell suspension reservoir. The effects of one or more test agents is assessed in each of these cell types by contacting the cells with the test agent to form a negative control mixture and a test mixture, directing the negative control mixture and the test mixture through a detection zone, and measuring the cellular responses of the negative control cells and the engineered or induced cells.

10 A difference in the response of the engineered or induced cells relative to the response of the negative control cells indicates that the test agent has an effect on the activity of the receptor.

The negative control cell is commonly referred to as the "host cell" and the engineered cell is commonly referred to as the "transfected cell" by those in the industry. To obtain engineered cells, the gene encoding the receptor may be introduced into a cell of the same type as the negative control cell using techniques such as transformation, calcium phosphate mediated transfection, electroporation, viral infection, transposition, or other techniques familiar to those skilled in the art. Expression of the receptor gene may be directed by a variety of vectors familiar to those skilled in the art. Alternatively, the cells may be induced to express the receptor through treatment with chemical agents or bioactive agents such as growth factors, cytokines, cell differentiation factors, or other agents known to those skilled in the art.

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EXAMPLE 6

The activity of a particular receptor may also be influenced by the characteristics of the particular cell type in which it is expressed. Thus, the same receptor may exhibit variations in activity between different cell types. The present invention may also be used to assess such cell type dependent differences in receptor activity. Each of the cell types to be assessed is placed in a cell suspension reservoir and the effects of one or more test agents on the activity of one or more receptors on the cells are determined by contacting the cells with one or more test agents to form test mixtures, directing the test mixtures through a detection zone, and measuring the cellular response of the cells as the test mixtures flow through the detection zone. The test agents may be receptor known receptor agonists, known antagonists, mixtures of a known agonist and a known antagonist, or compounds whose activity is unknown as described above. This process is performed for each of the cell types to be tested to determine the nature and magnitude of the cellular response in each cell type. To facilitate the analysis of multiple cell types, each of the cell types may be placed in one or

30 one or more reservoirs.

35 FIG. 13 shows an algorithm that may be used in the invention to perform cell mapping and cell receptor "fingerprinting". First, cells are mixed with standard agonist (step 1301) and the mixture is provided to a detector (step 1302). Next, the apparatus determines if this standard agonist, upon contact with the cells, triggers any cell response (step 1303). There are two possibilities, either the standard agonist does not produce any response (NO)

or it induces the cell response (YES). Cell response is determined by monitoring the signal from the detector for the particular standard agonist being evaluated. The control system first calibrates to establish a baseline and a maximal response, and any signal from the detector falling between those values is considered to be a positive response.

If the standard agonist causes no cell response, (NO), as measured by the monitoring part of the apparatus, 5 the system increments an agonist counter (step 1309) which indicates that a next standard agonist in a specified set of agonists will be tested with a particular cell line. The system then determines if all available agonists have been tested (step 1305). If all standard agonists have not been tested with a particular cell line, fresh cells from the original cell line are then supplied to the system (step 1306). These new cells are then automatically brought into contact with the next standard agonist from the predetermined set of agonist solutions (step 1301). Each 10 agonist solution in the set contains one or more ingredients that are known to initiate cell response through the stimulation of a known cell receptor, ion pump or ion channel molecules. The apparatus will keep repeating an admixture of different standard agonist substances with the cells until it detects that the cell response is triggered with a particular standard agonist, or until all agonists available to the machine have been tested.

If in step 1303, it is determined that a particular agonist triggers a cell response, the process is switched 15 over to the potency mode of action (step 1306) in which the cellular response at various concentration levels of the standard agonist is recorded. After step 1306 has been completed, the process then records the potency parameters, or profile, representing the level of cellular response produced in a given cell line at various concentration levels of the standard agonist (step 1307). The process then once again determines if all available standard agonists have been tested (step 1304). If all of the available agonists have been tested (YES), the system then determines if all 20 the available cell lines have been tested (step 1308). If the answer to this question is yes the process is complete (step 1312).

If in step 1308 it is determined that not all available cell lines have been tested, a cell counter is incremented (step 1310) which indicates that a next cell line in a specified set of cell lines will be tested. Next, the standard agonist counter is reset (step 1310) which indicates that the new cell line will be tested with all 25 available standard agonists, starting with a first designated agonist in the specified set of standard agonists. Cells from the new cell line are then supplied to the system (step 1311) and subsequently mixed with the specified agonist (step 1301). The above process steps 1301-1311 are then repeated. In one embodiment, the apparatus will keep repeating an admixture of different standard agonist substances with the cells until it detects that all agonists available to the apparatus have been tested with all cell lines available to the apparatus.

30 Fig. 14 shows an algorithm that may be used in the invention to perform transfected receptor characterization, particularly, of an orphaned receptor. First, in step 1401, an apparatus supplies cells (e.g., HEK293 cells) with a gene which encodes a receptor of interest. The apparatus then supplies the cells with a standard agonist (step 1402). The apparatus then monitors the signal from the detector (step 1403). The apparatus determines if this standard agonist, upon contact 35 with the cells, triggers any cell response (step 1404). There are two possibilities: either the standard agonist does not produce any response (NO), or it induces the cell response (YES). Cell response is determined by monitoring the signal from the detector for the particular standard agonist being evaluated. The control system first calibrates

establish a baseline and a maximal response, and any signal from the detector falling between those values is considered to be a positive response.

If the standard agonist causes no cell response, (NO), as measured by the monitoring part of the apparatus, the system increments a standard agonist counter (step 1405) which indicates that a next standard agonist in a specified set of standard agonists will be tested with the transfected cells. The system then determines whether all available standard agonists have already been tested with the transfected cells (step 1406). If not, the process moves back to step 1401 in which fresh cells from the same transfected cell line are automatically brought into contact with the next standard agonist from the predetermined set of agonist solutions (step 1402). Each standard agonist solution in the set contains one or more ingredients that are known to initiate cell response through the stimulation of a known cell receptor, ion pump or ion channel molecule. The apparatus will keep repeating an admixture of different standard agonist substances with the transfected cells until it detects that the cell response is triggered with a particular standard agonist, or until all agonists available to the machine have been tested.

If in step 1404 it is determined that the contact of the transfected cells with the standard agonist does not initiate the cell response, (YES), as measured by the monitoring part of the apparatus, the process then supplies host cells (step 1407) to be mixed with the same standard agonist (step 1408). This mixture is then supplied to the detector (step 1409) for measuring the cellular response produced by the standard agonist on the host cells. Next, the system determines if the standard agonist produces a cellular response in the host cell (step 1410). If a cell response is detected (YES) the system then increments the standard agonist counter (step 1405) after which the system determines if all available agonists have been tested (step 1410). If all available agonists have been tested, the process is complete (step 1412). If all available standard agonists have not been tested, the process moves back to step 1401 where fresh transfected cells are supplied to be mixed with the next standard agonist in the set of standard agonists (step 1402), and the process steps described above are repeated.

If in step 1410, no cell response is produced in the host cell by the standard agonist the process has detected a difference between the host cell and the transfected cell. At this point, the process is complete (step 1412). In one embodiment, the apparatus will keep repeating an admixture of different standard agonist substances with the cells until it detects that a particular agonist stimulates a response in the cells transfected with an orphaned receptor, and do not stimulate a response on host cells or until all standard solutions available to the machine have been tested.

A continuous flow diagram of the presently preferred cell mapping and cell receptor "fingerprinting" mode with the negative pressure fluidics system of Fig. 3 is shown on Figs. 15a - 15f. To practice this mode, diverting valve 347 is replaced by a multichannel diverting valve 347 which allows multiple reservoirs to be connected to the system.

STEP 1401: Transfected cells are switched over to a next cell reservoir.

The screening program starts from a state 1500 and enters state 1502 where it sets all parameters to their initial values. These initial parameters include, but are not limited to, zeroing counters, the internal initialization of software variables and subroutines.

Once the initial parameters are set, the software requests an operator to enter values for a maximum number of standard agonists, L_{MAX} , and a maximum number of cell lines, C_{MAX} , to be used in the experiment (Step 1503). The autosamplers 301 and 315 (Fig. 3) position the intake nozzles 307 and 323 at the corresponding "zero" positions occupied by a wash buffer reservoir 305 (Step 1504), and turns off proportioning valves 311 and 327, diverting valves 333, 335 and 347, as well as priming valves 313, 329, 341 and 345 (Step 1506). In a turned off state, priming valves 313, 329, 341 and 345, are normally opened, proportioning valves 311 and 327 connect, respectively, their outlets 312 and 328 with corresponding "normally opened" intake ports 309 and 325, diverting valve 335 connects its common outlet 334 with "normally opened" intake port 336, diverting valve 333 connects its common outlet 342 with the "normally opened" intake port 332, and multichannel diverting valve 347 connects its common outlet 346 with the first intake port 348 connected with the reservoir containing first cell suspension under investigation.

After the system has been initialized, pump 357 is started (Step 1508). This will force the wash buffer from the "zero" positioned reservoirs of both autosamplers 301 and 315 to run through nozzles 309 and 325 and outlet ports 312 and 328 of proportioning valves 311 and 327, respectively, priming valves 313 and 329, mixing zone 331, intake port 332 and outlet port 342 of diverting valve 333, priming valve 341, mixing zone 343, a reaction developing line 353, detector 355, pump 357 and then into a drain container 359. In mixing zone 343 this flow is mixed with the cell suspension 349 coming from intake tubing 348 connected with the first cell line, and an outlet port 346 of the multichannel diverting valve 347 through priming valve 345. Thus, the total flow passing mixing zone 343, is a sum of three flows, a first flow coming from the proportioning valve 311, a second flow coming from the valve 327 and a third flow coming from the multichannel diverting valve 347. During this step, both proportioning valves supply wash buffer 305 from the reservoirs located at "zero" position of each autosampler in such a way that the final flowing mixture consists of one part of cell suspension 349 and two parts of wash buffer 305.

After a delay determined in step 1509, which is needed for the flow of the mixture of cell suspension 349 and wash buffer 305 to stabilize, detector 355 registers a basal signal produced by the cells alone (Step 1510). After the basal signal is registered, it is saved as a reference signal, BS_1 (Step 1512) and computer 123 (Fig. 1) increments counter L (Step 1514).

The numerical content of the incremental counter L is increased by one each time it is triggered. The numeric value of the counter determines the position of the nozzle of intake port 323 of autosampler 315 that samples the sets of standard agonists 319 to be tested. Next, in step 1516, the numerical content, L_k , of the incremental counter L is compared with the previously entered (Step 1503) value of maximal number of agonists to be tested.

In step 1518, the nozzle 323 is moved to the position occupied by the standard agonist reservoir located at the L_k position of a rack of standard agonists 319 (Step 1518). Next, proportioning valve 327 is switched ON to open its "normally closed" intake port 323 to the common output port 328 (Step 1520). During Step 1520, the combined flow in mixing zone 343 and afterwards in detector 355 is composed of one portion

buffer 305 coming through nozzle 309 of proportioning valve 311, one portion of the standard agonist 319 to be tested, coming from nozzle 323 of proportioning valve 327 and one portion of particular cell suspension 349 coming from one of the multitude of the cell reservoirs through the intake tubing 348 to the output port 346 of multichannel diverting valve 347. After a delay provided by Step 1521, which is needed for the mixing process to stabilize, 5 detector 355 registers a standard agonist induced signal, SL_k , produced by given cells in the presence of the given standard agonist, L_k (Step 1522). After the SL_k signal is registered, its value is saved as a value, SL_k (Step 1523) and the apparatus proceeds to step 1530 where the proportioning valve 327 is turned OFF, thus connecting normally opened intake port 325 with buffer 305. In step 1532, autosampler 315 moves its nozzle 323 to a zero position occupied with reservoir filled with washing buffer. Proportioning valve 327 is turned ON (Step 1534), connecting 10 the normally closed inlet port 323 with outlet port 328. Delay, determined in step 1536, allows for the flow path composed of intake port 323 and outlet port 328 of the proportioning valve 327, priming valve 329, mixing zone 331, intake port 332 and outlet port 342 of the diverting valve 333, priming valve 341, mixing zone 343, reaction developing lines 353 and detector 355 to be washed clean of the remains of the previous compound. After the 15 delay, the proportioning valve 327 is turned OFF in the step 1538, thus bringing the system into original state..

Next, the value of the standard agonist induced signal, SL_k , is compared with the value of the reference basal signal, BS_i (Step 1544). If the SL_k signal is greater than the BS_i signal, the computer 123 (Fig. 1) will "flag" 20 the corresponding standard agonist as "positive" (Step 1540), which means that the particular cells express a receptor which is stimulated by the particular agonist. If SL_k is not greater than BS_i , computer 123 will "flag" the corresponding standard agonist as "negative" (Step 1542), which means that the particular cells do not contain a receptor which can be stimulated by the particular agonist.

Each time the condition, " $SL_k > BS_i$ ", is satisfied, the program flow will go through loop K-O initiating the gradient mode of action. In the gradient mode, step 1550 moves autosampler 315 at a position corresponding to the L_k value determined in step 1514. After that, the proportioning valve 327 is turned on to operate in the gradient mode (step 1552). In step 1554, the dose response curve is registered for the standard agonist stimulated 25 signal. Step 1556 calculates a potency parameter that could be determined as some specific concentration of the agonist causing a specific level of a signal. The data are saved in step 1558.

After the data are saved, the proportioning valve 327 is turned OFF (step 1560), autosampler 315 is moved to a zero position (step 1562), proportioning valve 327 is turned ON (step 1564) and, after a washing delay period determined in step 1566, the valve 327 is turned OFF again (step 1568). After step 1568 has been performed, the 30 process goes back, through line O, to the incremental counter L (step 1514).

If in step 1544, the SL_k signal is not larger than BS_i ..

then .. through line N to step 1514

If in step 1516 (Fig. 15b), L_k exceeds L_{max} , indicating that all agonists have been tested, the process moves 35 through line N to step 1570 were a computer 123 increments a cell counter C. The numeric value of the counter is compared (step 1572) with the maximum number of cells entered in step 1503. If the counter value, C, does not

exceed C_{MAX} , then controller L is set to zero in step 1574, multichannel diverting valve 347 is switched to the next position, connecting a new cell reservoir with the output 346, and the process returns to step 1509 (Fig. 15a) through line P. If in step 1572 the C_i value is higher than C_{MAX} , then the process moves to step 1578, where autosampler 315 is moved to its zero position and, proportioning valve 327 is turned ON (Step 1580). After a washing delay, determined in step 1582, the valve 327 is turned OFF (Step 1584) and pump 357 is turned OFF (Step 1586), and the whole program is stopped in step 1588.

5 A continuous flow diagram of the presently preferred orphaned receptor detection mode with the negative pressure fluidics system of Fig. 3 is shown on Figs. 16a - 16g. To practice this mode, diverting valve 347 is replaced by a multichannel diverting valve 347 which allows for at least two reservoirs 349, containing host cells, native or transfected with an empty vector, and cells transfected with the orphaned receptor of interest, to be connected separately to the common outlet 346.

10 15 The program begins from a start state 1600 and enters state 1602 where it sets all parameters to their initial values. These initial parameters include, but are not limited to, the internal initialization of software variables and subroutines. Then the program requests an operator to enter a maximum number of agonists to be tested (Step 1603).

Once the initial parameters are set up, the autosamplers 301 and 315 (Fig. 3) move their intake nozzles 307 and 323 to the corresponding "zero" positions occupied by a wash buffer reservoir 305 (Step 1604). Proportioning valves 311 and 327, diverting valves 333, 335 and 347, and priming valves 313, 329, 341 and 345 are turned OFF (Step 1606). In that turned off state, priming valves 313, 329, 341 and 345, are normally opened, 20 the outlets 312 and 328 of proportioning valves 311 and 327 are connected, respectively, with corresponding "normally opened" intake ports 309 and 325, outlet 334 of diverting valve 335 is connected with "normally opened" intake port 336, common outlet 342 of diverting valve 333 is connected with the "normally opened" intake port 332.

After the system has been initialized, diverting valve 347 is set to connect its common outlet 346 with the reservoir containing host cells, position 1, (Step 1608). The pump 357 is started to pump liquid (Step 1610). This will force the wash buffer from the "zero" positioned reservoirs of both autosamplers 301 and 315 to run through nozzles 309 and 325 and outlet ports 312 and 328 of proportioning valves 311 and 327, respectively, priming valves 313 and 329, mixing zone 331, intake port 332 and outlet port 342 of diverting valve 333, priming valve 341, 25 mixing zone 343, a reaction developing line 353, detector 355, pump 357 and into a drain container 359. In mixing zone 343, this flow is mixed with the host cell suspension coming from intake tubing 348 and an outlet port 346 of the multichannel diverting valve 347 through priming valve 345. Thus, the total flow passing through mixing zone 343 is a sum of three flows, a first flow coming from the proportioning valves 311 and 327, a second flow from the priming valves 313 and 329, and a third flow from the outlet port 346 of the multichannel diverting valve 347.

30 35 PROPORTIONING VALVE 311 SUPPLY WASHING BUFFER 305 AND RESERVOIRS LOCATED AT ZERO POSITION OF EACH AUTOSAMPLER SUCH A WAY THAT THE FINAL MIXTURE CONSISTS OF ONE PART OF THE HOST CELL SUSPENSION AND TWO PARTS OF WASHING BUFFER.

After a delay determined in step 1612, which is needed for the flow of the mixture of cell suspension and wash buffer to stabilize, detector 355 registers a basal signal produced by the host cells (Step 1614). After the basal signal is registered, it is saved as a reference signal, $(BS)_{HC}$ (Step 1616). In step 1620, multichannel diverting valve 347 is switched over to position 2 to supply the outlet 346 with the suspension of the transfected cells. After 5 a delay determined in step 1622, which is needed for the flow of the mixture of new cell suspension and wash buffer to stabilize, detector 355 registers a second basal signal produced by the transfected cells (Step 1624). After the basal signal is registered, it is saved as a reference signal, $(BS)_{TC}$ (Step 1626) and the pump is turned OFF (Step 1628). The computer 123 (Fig. 1) triggers the incremental counter L (Step 1630).

The numerical content of the incremental counter L is increased by one each time it is triggered, thus 10 determining the position of the nozzle of intake port 323 of autosampler 315 which samples the sets of agonists 319 to be tested. Next, the numerical content, L_k , of the incremental counter L is compared with the maximum number of the standard agonists to be tested, L_{max} (Step 1632).

If L_k does not exceed L_{max} , autosampler 315 positions nozzle 323 into the agonist reservoir located in the rack 319 at the position determined by the numerical value L_k (Step 1634). The pump 357 is turned ON in step 15 1636 and proportioning valve 327 is switched ON to open its "normally closed" intake port 323 to the common outlet port 328 (Step 1638) thus allowing the particular agonist flows through the priming valve 329, mixing zone 331 intake port 332 and outlet port 342 of the diverting valve 333, priming valve 341, mixing zone 343, where it is mixed with the suspension of transfected cells coming from outlet port 346 of multichannel diverting valve 347 and priming valve 345. After being mixed with the transfected cells in mixing zone 343, the liquid flow goes through 20 reaction developing lines 353, into detector 355 and further into waste reservoir 359. After a delay provided by Step 1640, which is needed for the mixing process to stabilize, detector 355 registers an agonist induced signal, $(SL_k)_{TC}$, produced by the particular orphaned receptor transfected cells in the presence of the given agonist, L_k (Step 1642). After the signal is registered, its value, $(SL_k)_{TC}$, is saved in step 1643 and the apparatus proceeds to step 1644 where proportioning valve 327 is turned OFF, thus connecting normally opened intake port 325 with buffer 25 305 to outlet port 328. In step 1646, autosampler 315 moves its nozzle 323 to position zero occupied with reservoir filled with washing buffer. Proportioning valve 327 is turned ON again (Step 1648) connecting inlet 323 with outlet 328. A delay, determined in step 1650, allows for the flow path, composed of intake port 323 and outlet port 328 of the proportioning valve 327, priming valve 329, mixing zone 331, intake port 332 and outlet port 342 of the diverting valve 333, priming valve 341, mixing zone 343, reaction developing lines 353, and detector 355 30 to be washed out of the remains of the agonist. After the delay (Step 1650), the proportioning valve 327 is turned OFF in step 1652 and the pump 357 is turned OFF in step 1654 thus ending the cycle.

After the cycle, the transfected cell suspension is compared with the value

corresponding reference basal signal, $(BS)_{TC}$ (step 1658). If the agonist induced signal, $(SL_k)_{TC}$, is not greater than the basal value, $(BS)_{TC}$, the computer 123 (Fig. 1) will "flag" the corresponding agonist, L_k as "negative" (Step 35 1660), which means that the cell does not contain a receptor which can be stimulated by the particular agonist, and the flow returns through line M back to the incremental counter L (Step 1630). If the $(SL_k)_{TC}$ signal is greater than

the $(BS)_{TC}$ signal, the computer 123 will "flag" the corresponding agonist as " $POSITIVE_{TC}$ " (Step 1656), which means that the transfected cells do express a receptor which can be stimulated by the particular agonist.

The next task is to determine if the host cells also express this receptor to distinguish it from the orphaned receptor which should be expressed only in transfected cells. To do so, after the standard agonist L_k has been flagged as positive in the step 1656, the program flow enters loop K - 0, where, in step 1662, the autosampler 315 moves its nozzle 323 to the position corresponding to the value obtained in counter L, L_k . Step 1664 switches diverting valve 347 to position 1, which connects host cell suspension reservoir 349 with mixing zone 343 through common output 346, through intake port 348 of the multichannel diverting valve 347, and through priming valve 345. Steps 1666 and 1668 turn valve 327 and pump 357 ON, respectively, allowing the buffer 305 coming from intake 309 of the autosampler 301, and standard agonist L_k , coming from the intake 323 and output port 328 of the proportioning valve 327, to be brought together in mixing zone 331 with the subsequent mixing of this solution with the host cells in the mixing zone 343. After a delay determined by step 1670, which is needed for the stream of the mixture of the cells and agonist to stabilize, detector 355 registers the agonist induced signal in the host cells, $(SL_k)_{HC}$ (Step 1672).

After the signal is registered, it is compared (Step 1674) with the corresponding basal signal, $(BS)_{HC}$, measured in step 1614. Depending on the results of the comparison, the agonist L_k is marked either as " $POSITIVE_{TC}/POSITIVE_{HC}$ " (Step 1676) or as " $POSITIVE_{TC}/NEGATIVE_{HC}$ " (Step 1678). In either case, the data are saved in step 1680 for further evaluation by the researcher.

After the data are saved, the multichannel diverting valve 347 is switched over to position 2 in step 1682, the proportioning valve 327 is turned OFF (step 1684), autosampler 315 is moved to the zero position (step 1686), proportioning valve 327 is turned ON (step 1688) and, after a washing delay, determined in step 1690, is turned OFF again (step 1692) and the pump 357 is turned OFF (Step 1694). After the step 1694 has been performed, the flow goes back to the incremental counter L (step 1630) through the line 0.

If in step 1632 (Fig. 16c), L_k does exceed L_{max} , then the process moves to step 1700 (Fig. 16g) where the counter L is set to zero. Next, the autosampler 315 is moved to the zero position in step 1702, the proportioning valve 327 is turned ON in step 1704 and after a washing delay determined in step 1706, both proportioning valve 327 and pump 357 are turned OFF in steps 1708 and 1710 respectively, after which the program stops in step 1712.

Although the invention has been described in detail with reference to certain particular embodiments thereof, it will be understood that any variations and modifications apparent to those of skill in the art will still fall within the spirit and scope of the invention. Other embodiments not specifically described herein are also included.

WHAT IS CLAIMED IS:

1. A method for identifying compounds having biological activity, comprising the steps of:
 - (a) combining a homogeneous suspension of living cells with a test compound having an unknown cellular effect to form a test mixture;
 - 5 (b) directing the test mixture through a detection zone; and
 - (c) measuring a cellular response of said suspended cells to said test compound as said test mixture is flowing through said detection zone.
2. The method of Claim 1, further comprising the steps of:
 - (d) combining a homogeneous suspension of said cells with a standard compound having a known 10 effect on said cellular response of said cells to form a standard mixture;
 - (e) directing the standard mixture through the detection zone; and
 - (f) measuring the cellular response of said cells to said standard compound.
3. The method of Claim 2, wherein said standard compound and said test compound are simultaneously mixed with said cells in said combining steps, and said measuring step detects said known effect or 15 an alteration of said known effect.
4. The method of Claim 3, wherein said standard compound is an antagonist of said cellular response.
5. The method of Claim 3, wherein said standard compound is an agonist of said cellular response.
6. The method of Claim 3, in which steps (a) and (d) are performed simultaneously; steps (b) and (e) 20 are performed simultaneously; and steps (c) and (f) are performed simultaneously using a single suspension of said cells.
7. The method of Claim 3, in which steps (a), (b), and (c) are performed first, and then steps (d), (e), and (f) are performed, wherein the test compound is added together with the standard compound in step (d).
8. The method of Claim 7, in which said cellular response is detected in step (c) to indicate that said 25 test compound is active to generate said response, and in which said standard compound is an antagonist, whereby a decrease in said cell response from step (c) to step (f) is indicative that said test compound is an agonist of said known effect.
9. The method of Claim 7, in which said cellular response is not detected in step (c), indicating that said test compound is not active to generate said response, and in which said standard compound is an agonist, whereby an alteration of said known effect detected in step (f) is indicative that said test compound is an antagonist 30 of said known effect.
10. The method of Claim 7, performed automatically, in which
a plurality of successive series of antagonists are automatically added as said standard compound in step (d) if said cellular response is detected in step (c) to indicate that said test compound is active to generate said cellular response, whereby a decrease in said cellular response detected in step (f) is indicative that said test compound is an agonist of said known effect; and
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in which a series of agonists are automatically added as said standard compound in step (d) when said cellular response is not detected in step (c), whereby an alteration of said known effect detected in step (f) is indicative that said test compound is an antagonist of said known effect.

11. The method of Claim 10, further comprising the steps of:

5 if step (f) indicates that said compound is an agonist of said known effect, then automatically determining the concentration dependence of agonist activity of said test compound by repeating steps (a), (b), and (c), and (d), (e), and (f) while varying the concentration of said test compound and said standard compound and recording resultant changes in said cellular response; and

10 if step (f) indicates that said compound is an antagonist, then automatically determining the concentration dependence of inhibition of said cellular response in the presence of said agonistic standard compound by repeating steps (d), (e), and (f) while varying the concentration of said test compound and said standard compound and recording resultant changes in said cellular response.

12. The method of Claim 11, further comprising the step when step (f) indicates that said compound is an antagonist of:

15 (g) automatically determining the concentration dependence of cell response activation by repeating steps (d), (e), and (f) for a zero concentration of said test compound while varying the concentration of said standard compound and recording resultant changes in cellular response, and then repeating this step (g) for different concentrations of said test compound.

13. The method of Claim 11, further comprising the step when step (f) indicates that said compound is an agonist, of:

(h) automatically determining the concentration dependence of cell response activation by repeating steps (d), (e), and (f) for a zero concentration of said standard compound while varying the concentration of said test compound and recording resultant changes in cellular response, and then repeating this step (h) for different concentrations of said compound.

25 14. The method of Claim 11, in which the varying of the concentration of the test compound is done continuously.

15. The method of Claim 11, in which the varying of the concentration of the test compound is done stepwise.

16. The method of Claim 11, in which the varying of the concentration of the standard compound is 30 done continuously.

17. The method of Claim 11, in which the varying of the concentration of the standard compound is

35 18. The method of Claim 11, further comprising the step of graphically displaying the recorded changes in said cellular response.

19. The method of Claim 1, wherein said cellular response is a change in intracellular ion concentration.

20. The method of Claim 19, wherein said ion is calcium, magnesium, proton, sodium, or potassium.

21. The method of Claim 9, wherein said ion is detected using an intracellular dye.
22. The method of Claim 21, wherein said dye is fluorescent.
23. The method of Claim 1, further comprising the step of repeating steps (a)-(c) on each of the cell types in a series of cell types to be tested until the effect of said test compound has been measured in each cell type of said series of cell types.
5
24. The method of Claim 23, further comprising the steps of:
combining a homogenous suspension of each of said cell types in said series of cell types with a standard compound having a known effect on said cellular response of said cells to form a series of standard mixtures;
- 10 directing said series of standard mixtures through the detection zone; and
measuring the cellular response of each of said cell types to said standard compound.
25. The method of Claim 23 wherein the standard compound and the test compound are simultaneously mixed with said cells in said combining steps and said measuring step detects said known effect or an alteration of said known effect.
15
26. The method of Claim 25 wherein said standard compound is an antagonist of said cellular response.
27. The method of Claim 26 wherein said standard compound is an agonist of said cellular response.
28. An apparatus for automatically measuring the effect of a plurality of test compounds on living cells, comprising:
20 a test compound sampler for sequentially providing samples of multiple test compounds;
a cell suspension input for providing a suspension of living cells;
a mixing zone, coupled to the test compound sampler, for receiving the samples of the test compounds from the test compound sampler, receiving the suspension of living cells from the cell suspension input and mixing each test compound with the suspension of living cells; and
- 25 a detector, coupled to the mixing zone, for measuring a cellular response of the suspended cells to each test compound.
29. The apparatus of Claim 28 further comprising a standard compound sampler, coupled to said mixing zone, for providing a sample of a standard compound having a known effect on said cellular response of said suspended cells, wherein said mixing zone receives the sample of the standard compound from the standard compound sampler and mixes the standard compound with the suspended cells and said detector measures the cellular response of the suspended cells to the standard compound.
30
- 35 31. The apparatus of Claim 29 further comprising:
a simultaneous mixing zone, for receiving the samples of the test compounds from the test compound sampler and the sample of the standard compound from the standard compound sampler and mixing each test compound with the sample of the standard compound; and
a detector, coupled to the simultaneous mixing zone, for measuring a cellular response of the suspended cells to each test compound and said detector detects said known effect or an alteration of said known effect.

a first gradient device, coupled to said test compound sampler, for automatically adjusting the concentration level of said test compound transferred to said mixing zone from said test compound sampler; and

5 a second gradient device, coupled to said standard compound sampler, for automatically adjusting the concentration level of said standard compound transferred to said mixing zone from said standard compound sampler.

10 32. The apparatus of Claim 31 further comprising a switching valve, coupled to the first and second gradient devices at an input of the switching valve and coupled to said mixing zone at an output of the switching valve, for selectively switching the flow of a concentration of said test compound or a concentration of said standard compound or both to the mixing zone where the test compound and/or the standard compound is then mixed with said suspension of cells.

15 33. The apparatus of Claim 32 further comprising a calibration unit, coupled to said switching valve, wherein the switching valve also selectively switches the flow of a calibration solution provided by the calibration unit into said mixing zone where the calibration solution is mixed with said suspension of cells.

20 34. The apparatus of Claim 33 further comprising reaction developing lines coupled to the output of said mixing zone, for receiving a mixture of said cell suspension mixed with either said test compound, said standard compound or said calibration solution, and providing a flow path for the mixture such that there is adequate time for the suspension cells to react with the test compound, the standard compound or the calibration solution, wherein the reaction developing lines is further coupled to the input of said detector which receives the mixture from the reaction developing lines.

25 35. The apparatus of Claim 34 wherein said detector detects changes in intracellular ion concentration.

36. The apparatus of Claim 35 wherein said ion is calcium, magnesium, proton, sodium, or potassium.

37. The apparatus of Claim 35 wherein said detector detects changes in intracellular ion concentration using an intracellular dye.

25 38. The apparatus of Claim 37 wherein said dye is fluorescent.

39. The apparatus of Claim 34 further comprising:

30 a controller, coupled to said first and second gradient devices, said test compound sampler, said standard compound sampler and said switching valve, for controlling their operation; and

a computer, coupled to the controller, for sending command signals to the controller in accordance

35 with a software program implemented by the computer, wherein the computer is also coupled to said detector in order to send and receive cell response measurement signals to and from the detector.

40 The apparatus

41 of claim 34, wherein the apparatus further comprises a computer for sending command signals to the controller in accordance with a software program implemented by the computer, wherein the computer is also coupled to said detector in order to send and receive cell response measurement signals to and from the detector.

42 The apparatus of claim 41, wherein the computer is a personal computer.

35 43. The apparatus of claim 40 further comprising:
a controller, coupled to said test compound sampler, for controlling the operation of the test compound sampler; and

-46-

a computer, coupled to the controller, for sending command signals to the controller in accordance with a software program implemented by the computer, thereby controlling the selection and retrieval of test compounds by the test compound sampler from said test compound library.

42. The apparatus of Claim 41 further comprising a gradient pump having an input and an output, 5 coupled to said test compound sampler, for adjusting the concentration level of said test compound transferred to said mixing zone from said test compound sampler, wherein:

said test compound sampler comprises:

a first intake nozzle for receiving said specified test compound;

a second intake nozzle for receiving a buffer solution; and

10 wherein said gradient pump is coupled to the first and second intake nozzles and receives specified concentrations of the test compound by adjusting the amount of test compound and buffer solution received by the first and second intake nozzles, respectively, wherein the buffer solution is a diluting agent of the test compound.

43. The apparatus of Claim 42 further comprising a standard compound sampler for providing a sample 15 of a standard compound to said mixing zone.

44. The apparatus of Claim 43 wherein said standard compound sampler is an automated robotic sampler capable of selecting a specified standard compound from a library of standard compounds.

45. The apparatus of Claim 44 further comprising a second gradient pump having an input and an output, coupled to said standard compound sampler, for adjusting the concentration level of said standard compound 20 provided to said mixing zone from said standard compound sampler, wherein:

said standard compound sampler comprises:

a third intake nozzle for receiving said specified standard compound;

a fourth intake nozzle for receiving a buffer solution; and

25 wherein said second gradient pump is coupled to the third and fourth intake nozzles and receives specified concentrations of the standard compound by adjusting the amount of standard compound and buffer solution received by the third and fourth intake nozzles, respectively, wherein the buffer solution is a diluting agent of the standard compound.

46. The apparatus of Claim 45 further comprising a second mixing zone coupled to the outputs of the first and second gradient pumps, for receiving and mixing said specified concentrations of said specified test 30 compound and said specified standard compound, such that the output of the second mixing zone is provided to said first mixing zone.

Apparatus for receiving a test compound

calibration unit for providing a calibration solution; and

35 a switching valve, having a first input coupled to said second mixing zone, a second input coupled to the calibration unit, and an output coupled to said first mixing zone, for switching between the flow of

either a compound mixture from the second mixing zone or the calibration solution from the calibration unit and then providing the flow to the first mixing zone where it may be mixed with said cell suspension.

48. The apparatus of Claim 47 wherein said calibration unit comprises:

5 a calibration maximum solution which provides for maximal cell response when mixed with said cell suspension;

a calibration minimum solution which provides for minimal cell response when mixed with said cell suspension;

10 a diverting valve having a first input coupled to the calibration maximum solution and a second input coupled to the calibration minimum solution, for switching between the flow of either the calibration maximum solution or calibration minimum solution; and

15 a pump, coupled to the output of the diverting valve and an input of said switching valve, for pumping either the calibration maximum or calibration minimum solution from the diverting valve into the switching valve.

49. The apparatus of Claim 48 further comprising a second pump, coupled to an input of said first mixing zone, for pumping said suspension of cells from said cell suspension input into the first mixing zone.

50. The apparatus of Claim 49 further comprising a reaction developing line, having an input coupled to an output of said first mixing zone and an output coupled to an input of said detector, for providing a flow path and a reaction time delay for a mixture received from the first mixing zone and for providing the mixture to the detector.

20 51. The apparatus of Claim 50 further comprising:

a controller, coupled to said first and second gradient pumps, said test compound sampler, said standard compound sampler and said switching valve, said first and second mixing zones, said first and second pumps and said diverting valve for controlling their operation; and

25 a computer, coupled to the controller, for sending command signals to the controller in accordance with a software program implemented by the computer, wherein the computer is also coupled to said detector in order to send and receive cell response measurement signals to and from the detector.

52. The apparatus of Claim 51 wherein said detector detects changes in intracellular ion concentration.

53. The apparatus of Claim 52 wherein said ion is calcium, magnesium, proton, sodium, or potassium.

30 54. The apparatus of Claim 52 wherein said detector detects changes in intracellular ion concentration using an intracellular dye.

- apparatus of claim 48 further comprising

35 a pump, coupled to the output of said detector, for providing negative pressure to the apparatus.

a proportionating valve, coupled to said test compound sampler, for adjusting the concentration level of said test compound transferred to said mixing zone from said test compound sampler, wherein said test compound sampler further comprises

a first intake nozzle for receiving said specified test compound;
a second intake nozzle for receiving a buffer solution; and
the proportionating valve receives specified concentrations of the test compound by adjusting the amount of test compound and buffer solution received by the first and second intake nozzles, respectively,
5 wherein the buffer solution is a diluting agent of the test compound.

57. The apparatus of Claim 56 further comprising:
an automated standard compound sampler capable of selecting a specified standard compound from a library of standard compounds, said standard compound sampler including a third intake nozzle for receiving said specified standard compound and a fourth intake nozzle for receiving a buffer solution; and
10 a second proportionating valve, coupled to the third and fourth intake nozzles, for receiving specified concentrations of the standard compound by adjusting the amount of standard compound and buffer solution received by the third and fourth intake nozzles, respectively, wherein the buffer solution is a diluting agent of the standard compound.

58. The apparatus of Claim 57 further comprising:
15 a first priming valve, coupled to the output of said first proportionating valve, for receiving said specified concentration of said test compound and providing the test compound to said mixing zone;
a second priming valve, coupled to the output of said second proportionating valve, for receiving said specified concentration of said standard compound and providing the standard compound to the mixing zone.
20

59. The apparatus of Claim 58 further comprising:
a calibration unit including a calibration maximum solution which provides for maximal cell response when mixed with said cell suspension and a calibration minimum solution which provides for minimal cell response when mixed with the cell suspension;
25 a first diverting valve, having a first input coupled to the calibration maximum solution and a second input coupled to the calibration minimum solution, for switching between the flow of either the calibration maximum solution or calibration minimum solution;
a second diverting valve, having a first input coupled to the output of said mixing zone and a second input coupled to the output of said first diverting valve, for switching between the flow of either a calibration solution from the first diverting valve or a mixture from the mixing zone;
30 a third priming valve, coupled to the output of the second diverting valve, for receiving a mixture from the second diverting valve; and

coupling the third priming valve with said cell suspension, wherein said cell suspension input and said detector are coupled to the second mixing zone instead of the first mixing zone.

35 60. The apparatus of Claim 59 further comprising a reaction developing line, having an input coupled to the output of said second mixing zone and an output coupled to an input of said detector, for providing a flow

path and a reaction time delay for a mixture received from the second mixing zone before the mixture reaches said detector.

61. The apparatus of Claim 60 wherein said cell suspension input comprises:
 - 5 a cell suspension reservoir;
 - a buffer reservoir;
 - a third diverting valve, having a first input coupled to the cell suspension reservoir and a second input coupled to the buffer reservoir, for adjusting the concentration of the cell suspension, wherein the buffer is a diluting agent of the cell suspension; and
 - 10 a fourth priming valve, coupled to the output of the third diverting valve, for receiving the cell suspension mixture from the third diverting valve and providing this mixture to said second mixing zone.
62. The apparatus of Claim 61 wherein said detector detects changes in intracellular ion concentration.
63. The apparatus of Claim 62 wherein said ion is calcium, magnesium, proton, sodium, or potassium.
64. The apparatus of Claim 62 wherein said detector detects changes in intracellular ion concentration using an intracellular dye.
- 15 65. The apparatus of Claim 64 wherein said dye is fluorescent.
66. The apparatus of Claim 61 further comprising a plurality of cell suspension reservoirs.
67. A method of characterizing the receptors present in a cell comprising the steps of:
 - (a) combining a suspension of cells with a test agent known to influence the activity of a particular receptor to form a test mixture;
 - 20 (b) directing said test mixture through a detection zone;
 - (c) measuring a cellular response of said suspension of living cells to said test agent as said test mixture is flowing through said detection zone wherein a response to a test agent indicates that said cell expresses a receptor known to respond to said test agent; and
 - (d) repeating steps (a)-(c) with a series of test agents until the effects of each test agent has been measured.
- 25 68. The method of Claim 67, wherein the test agent comprises an agonist.
69. The method of Claim 67 wherein the test agent comprises an antagonist.
70. The method of Claim 67 wherein the test agent comprises a mixture of an antagonist and an agonist.
- 30 71. The method of Claim 67 further comprising repeating steps (a)-(d) on a series of different cell types to determine the receptors expressed by each cell type

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contacting a negative control cell type which lacks said receptor with said test agent to form a negative control mixture;

36 directing said negative control mixture through a detection zone

measuring the cellular response of said suspension of living cells to said test compound as said negative control mixture is flowing through said detection zone;

contacting cells of the same cell type as the negative control which have been engineered or induced to express said receptor with said test agent to form a test mixture;

5

directing said test mixture through a detection zone;

measuring the cellular response of the cells in the test mixture to said test agent, whereby a difference in the measured response of said cells in said test mixture relative to the measured response of said negative control cells indicates that said test agent has an effect on the activity of said receptor.

73. The method of Claim 72, wherein said test agent comprises an agonist.

10

74. The method of Claim 72, wherein said test agent comprises an antagonist.

75. The method of Claim 72, wherein said test agent comprises a mixture of an agonist and an antagonist.

15

76. A method of determining the activity of one or more receptors in a series of cell types comprising:

(a) combining a suspension of living cells comprising a member of said series of cell types with an agent known to influence the activity of a particular receptor to form a test mixture;

(b) directing the test mixture through a detection zone;

(c) measuring the cellular response of said suspension of living cells to said test agent;

(d) repeating steps (a)-(c) on each member of the series of cell types until the effect of said test agent has been measured in each cell type of said series.

20

77. The method of Claim 76, wherein said test agent comprises a known receptor agonist.

78. The method of Claim 77, wherein said test agent comprises a known receptor antagonist.

79. The method of Claim 77, wherein the test agent comprises a mixture of a known agonist and a known antagonist.

25

80. The method of Claim 77, wherein the test agent comprises a compound whose activity is unknown.

81. The method of Claim 77 wherein the test agent comprises a mixture of a compound whose activity is unknown and a compound which is a known agonist or a known antagonist.

Fig. 1

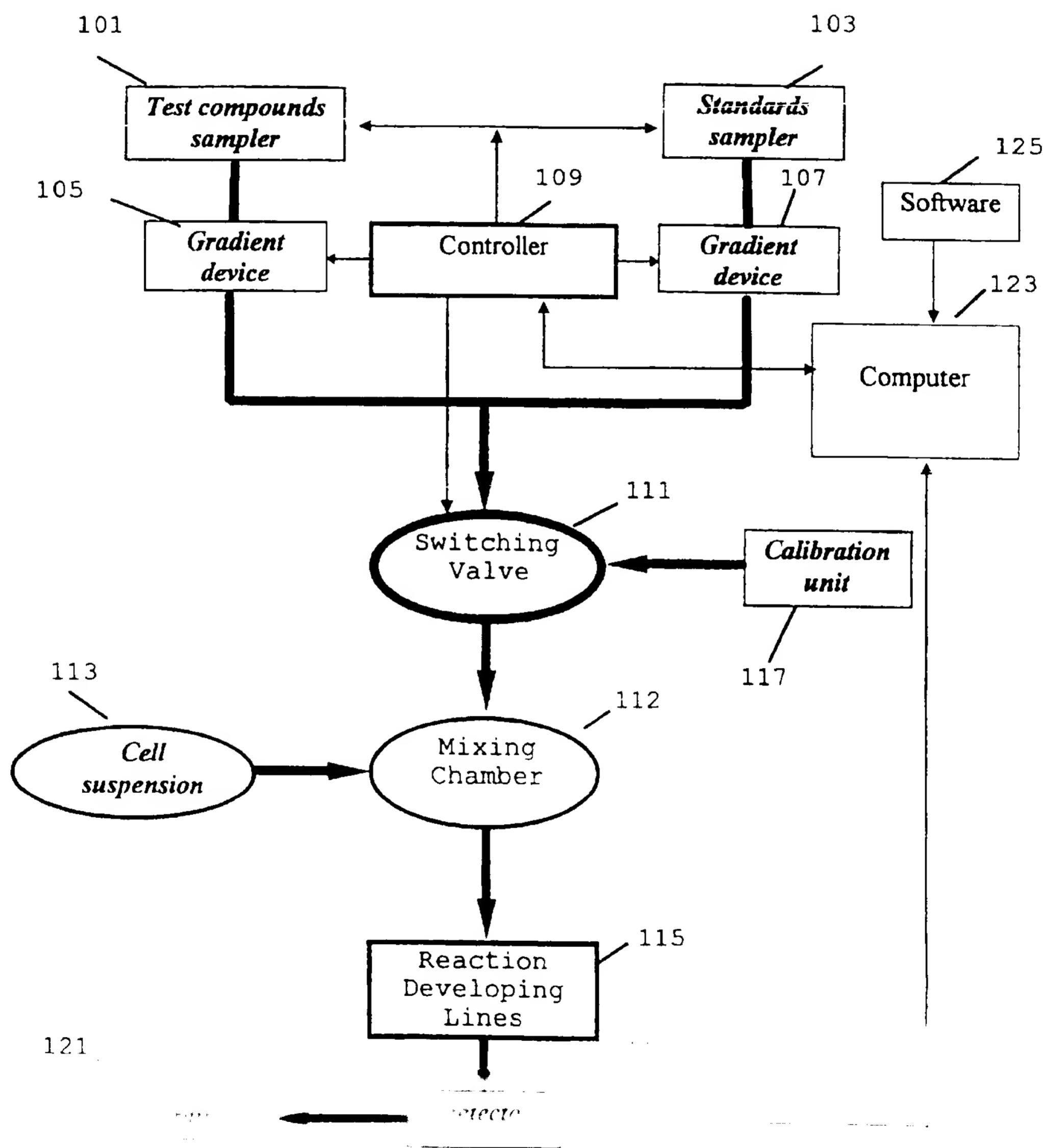


Fig.2

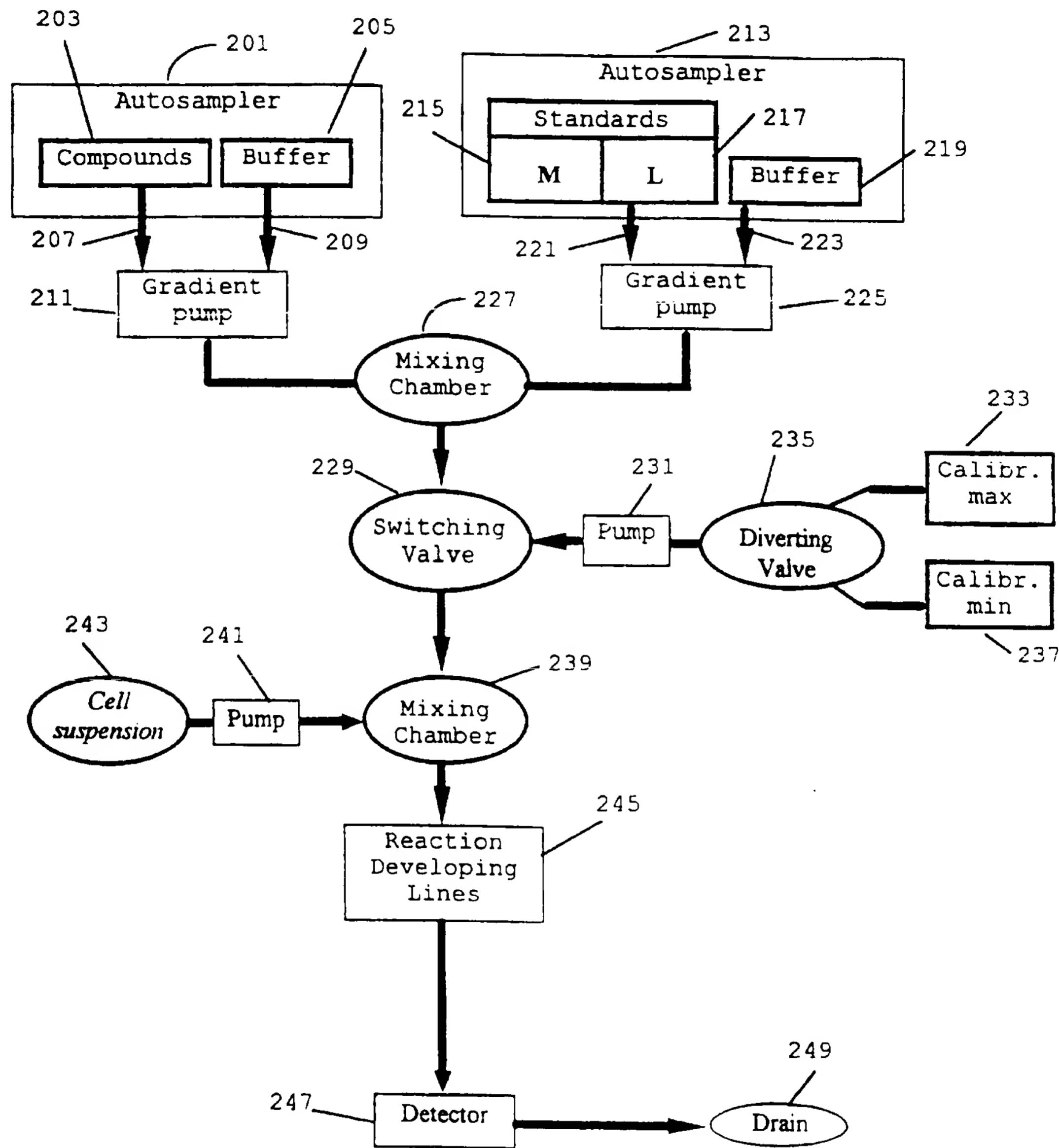


Fig.3

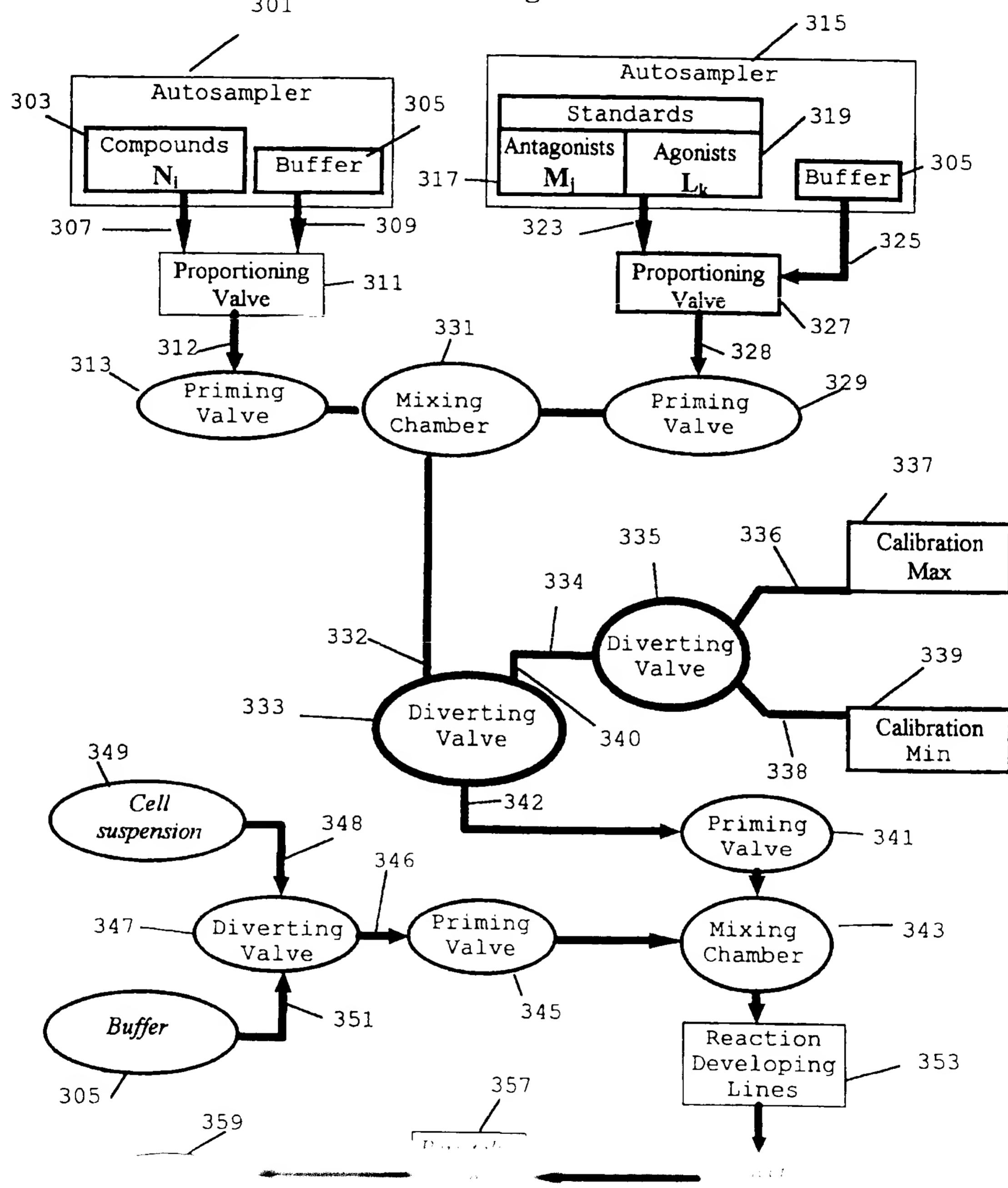


Fig. 4

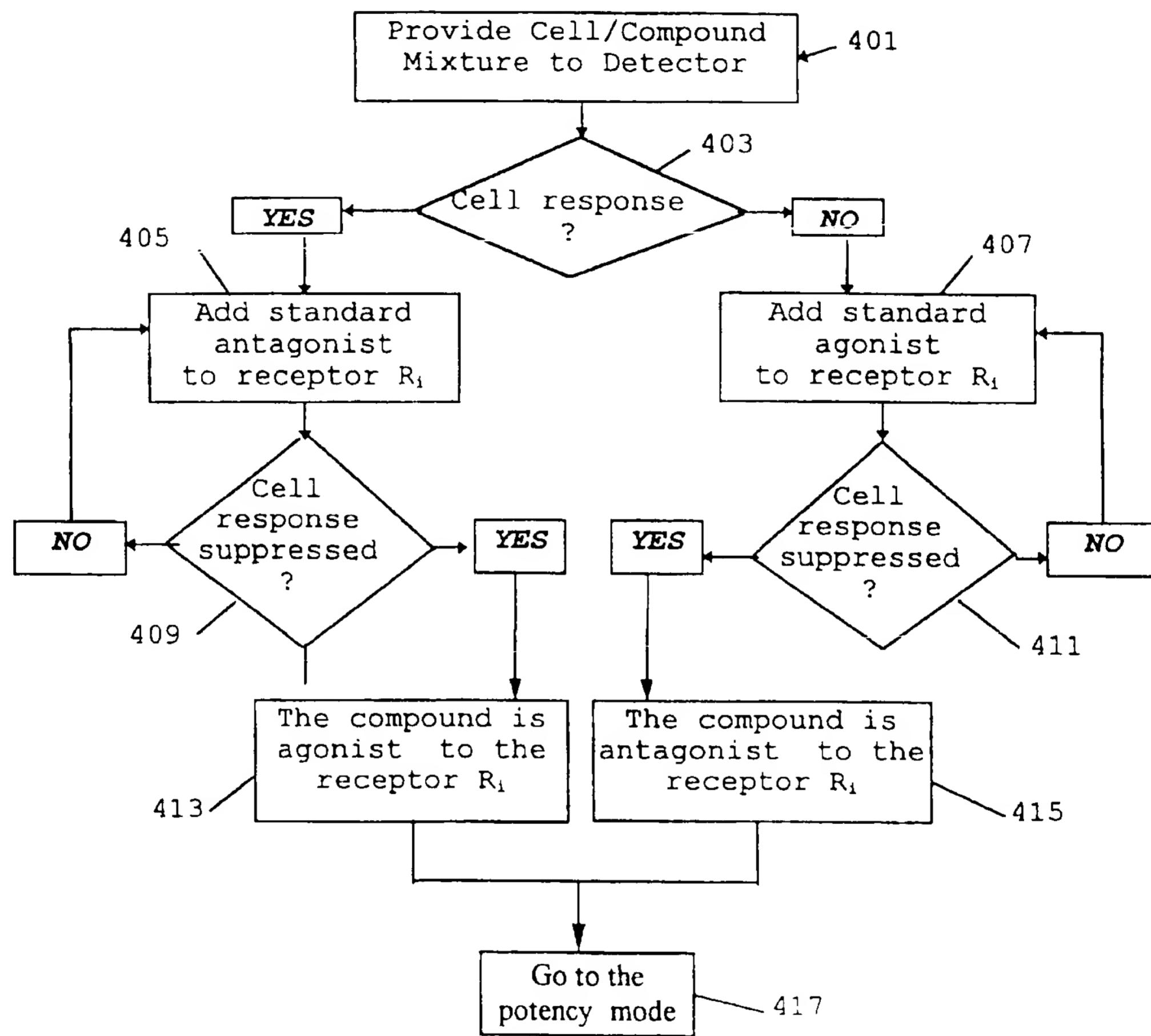
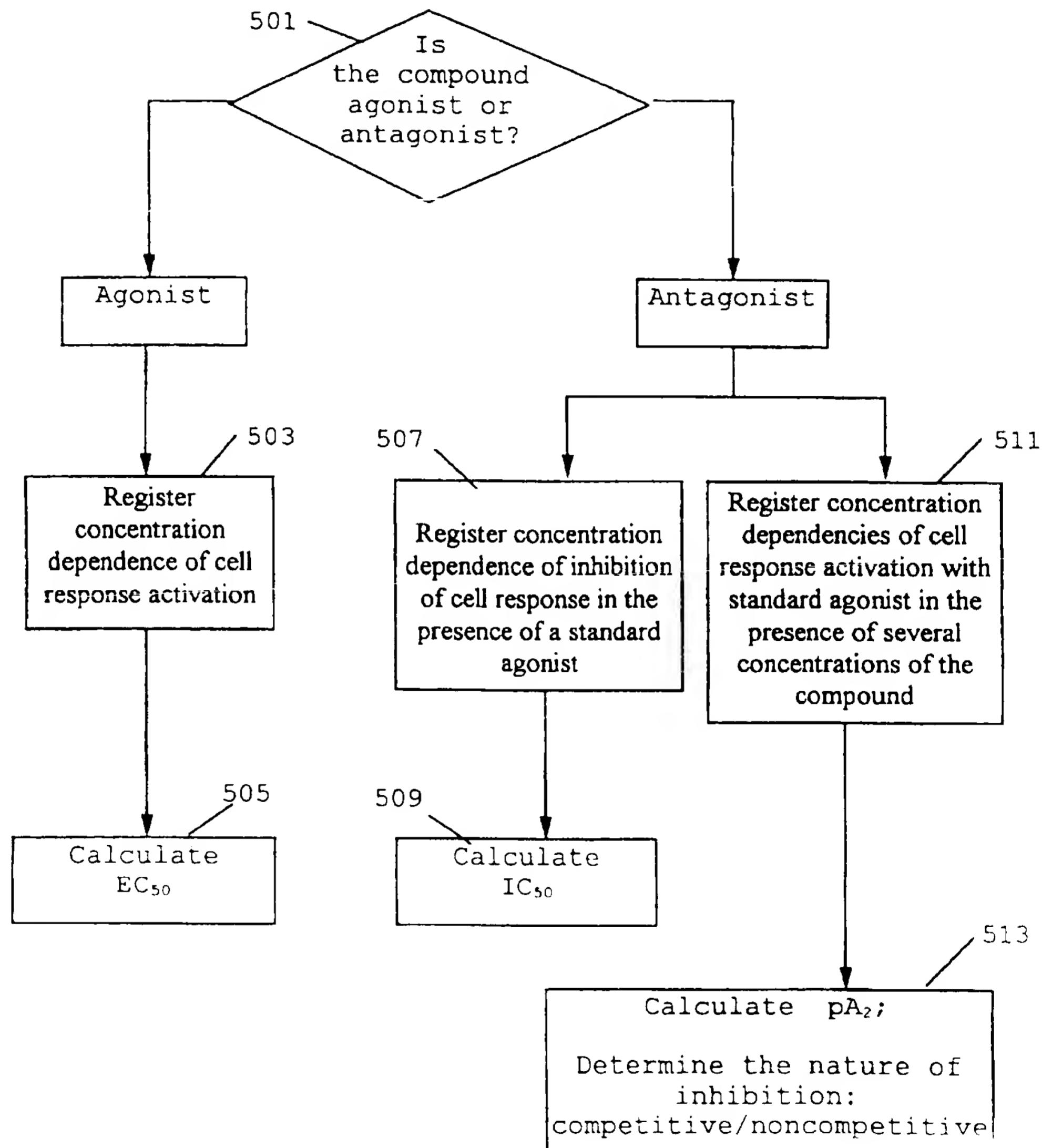


Fig. 5



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Fig. 6

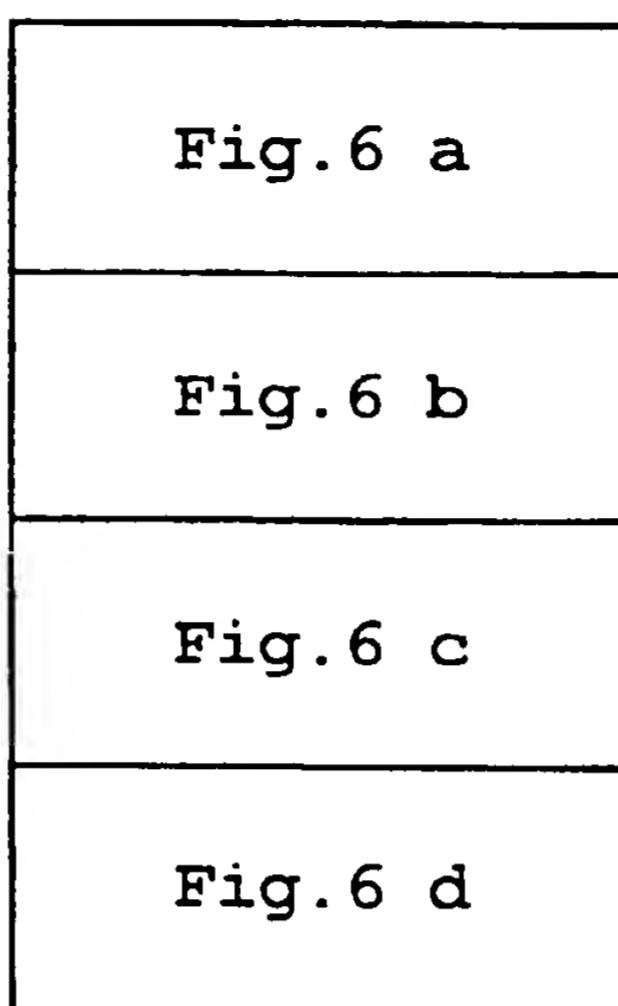
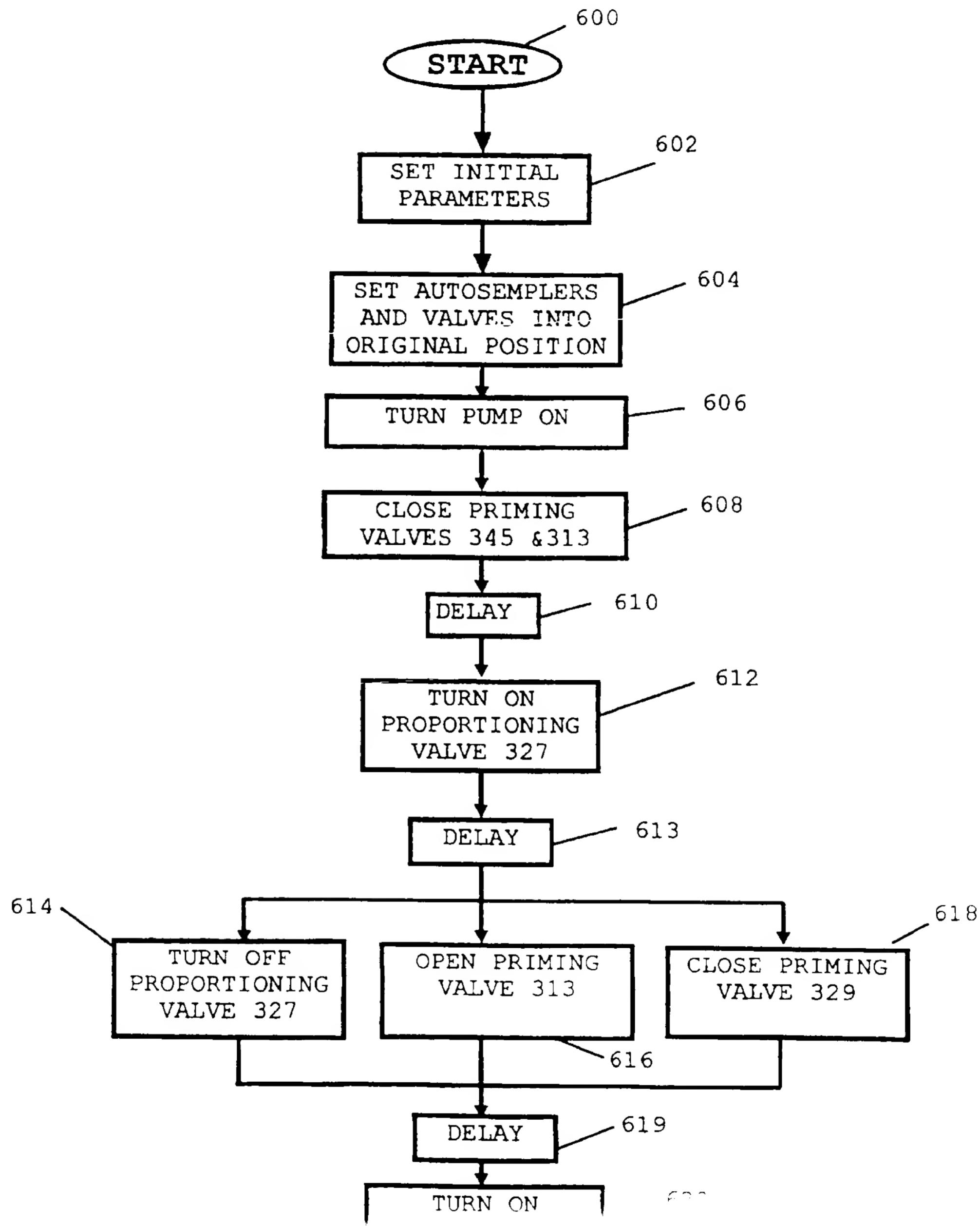
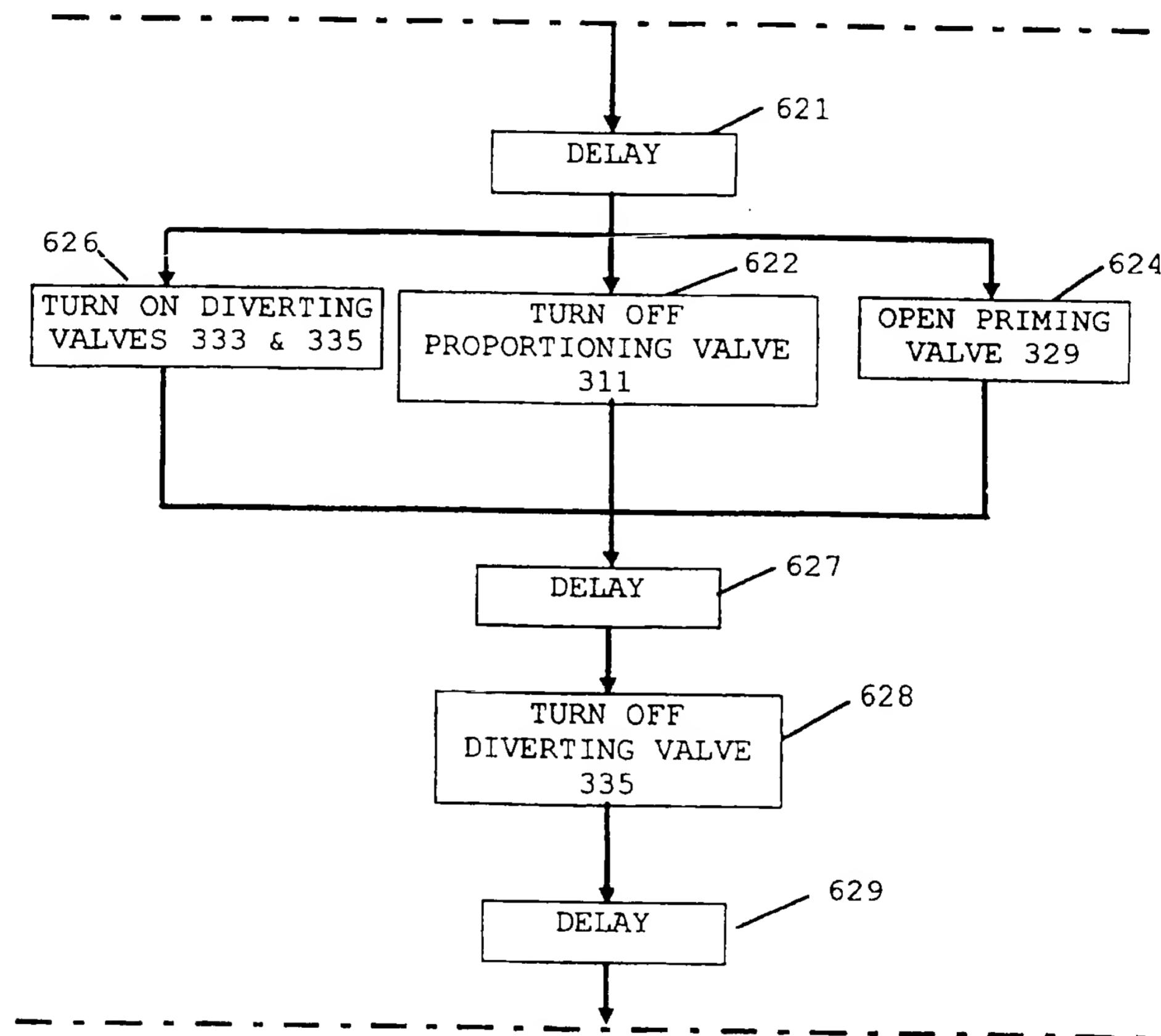


Fig. 6a



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Fig. 6b



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Fig. 6c

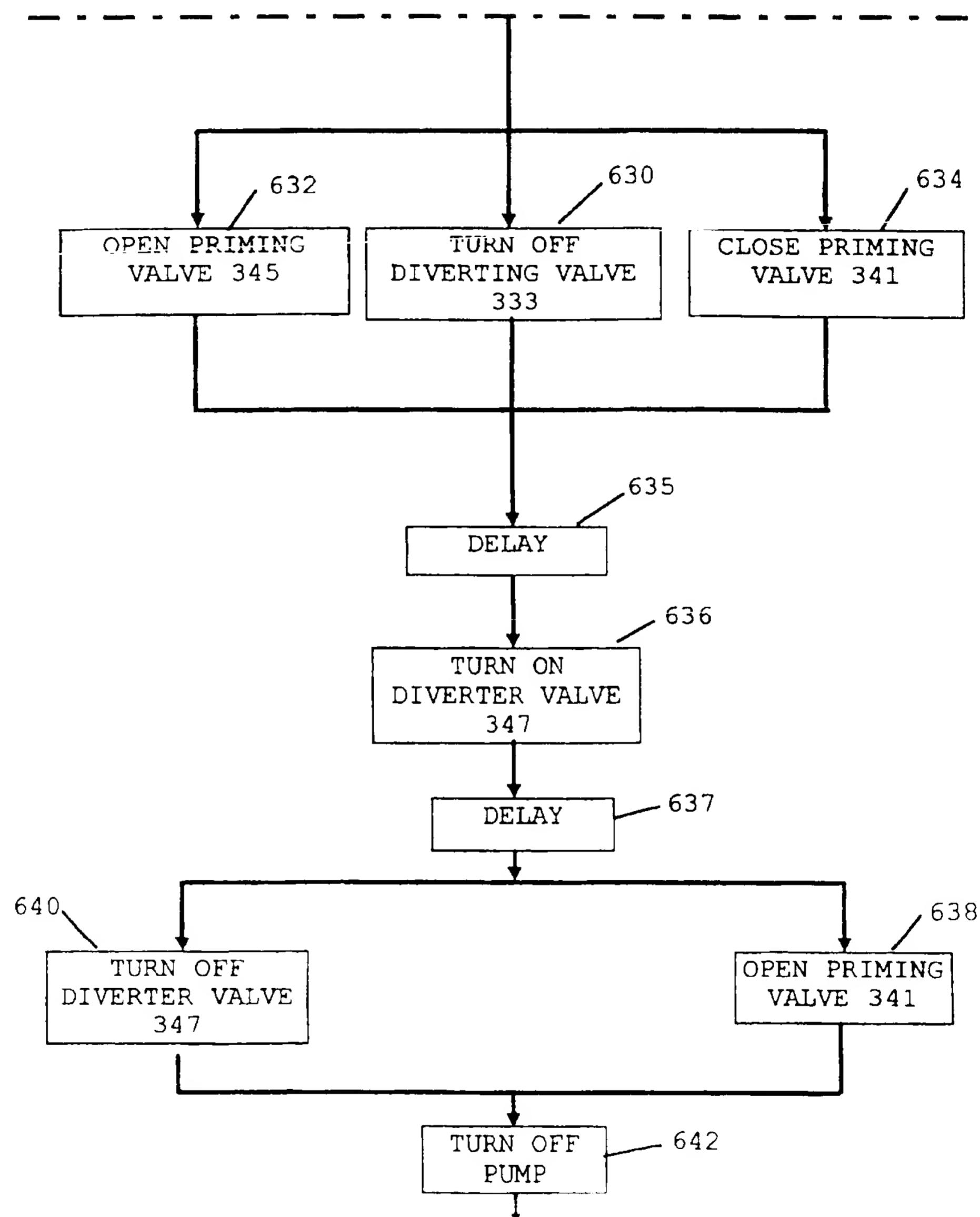
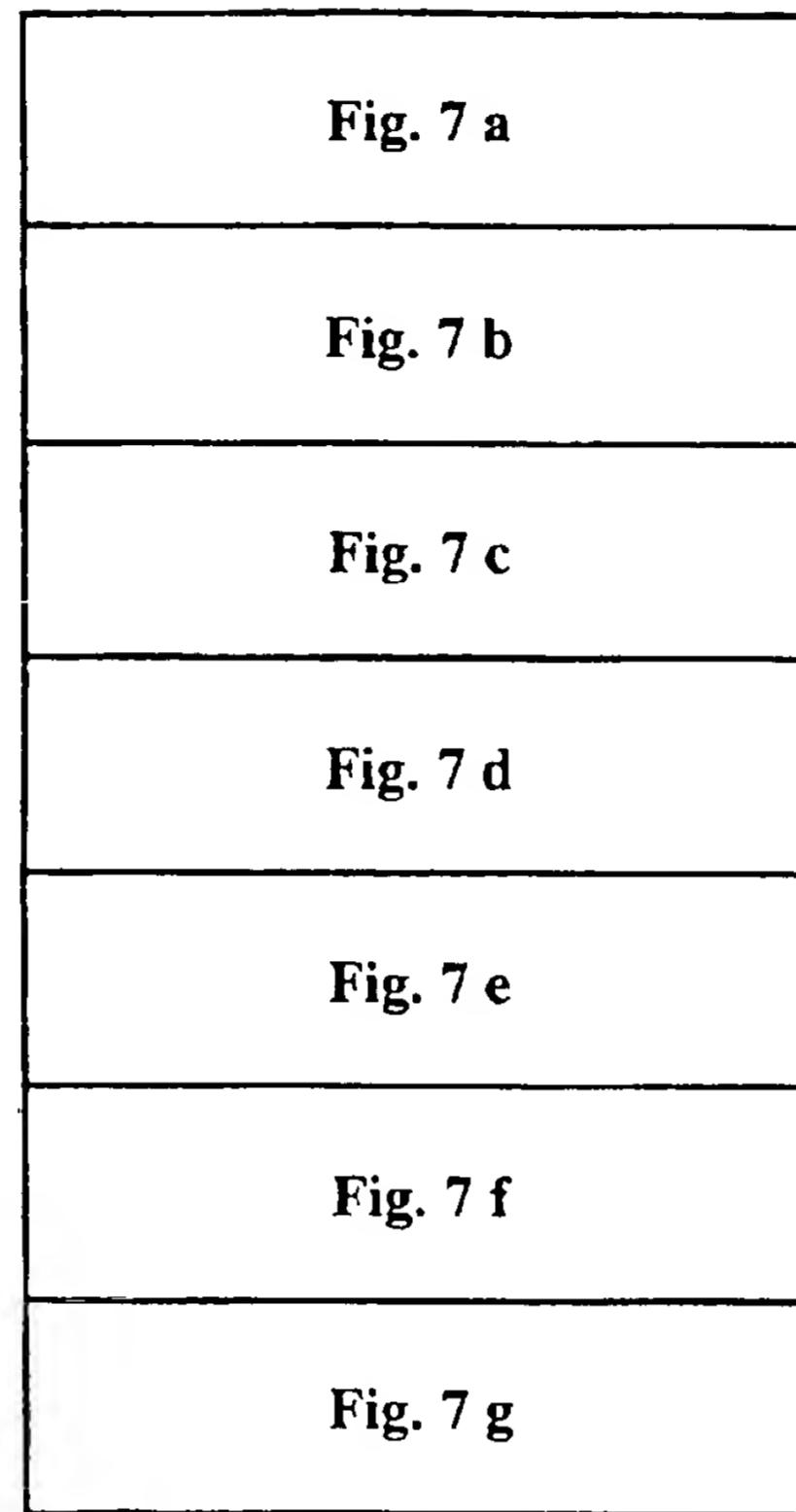


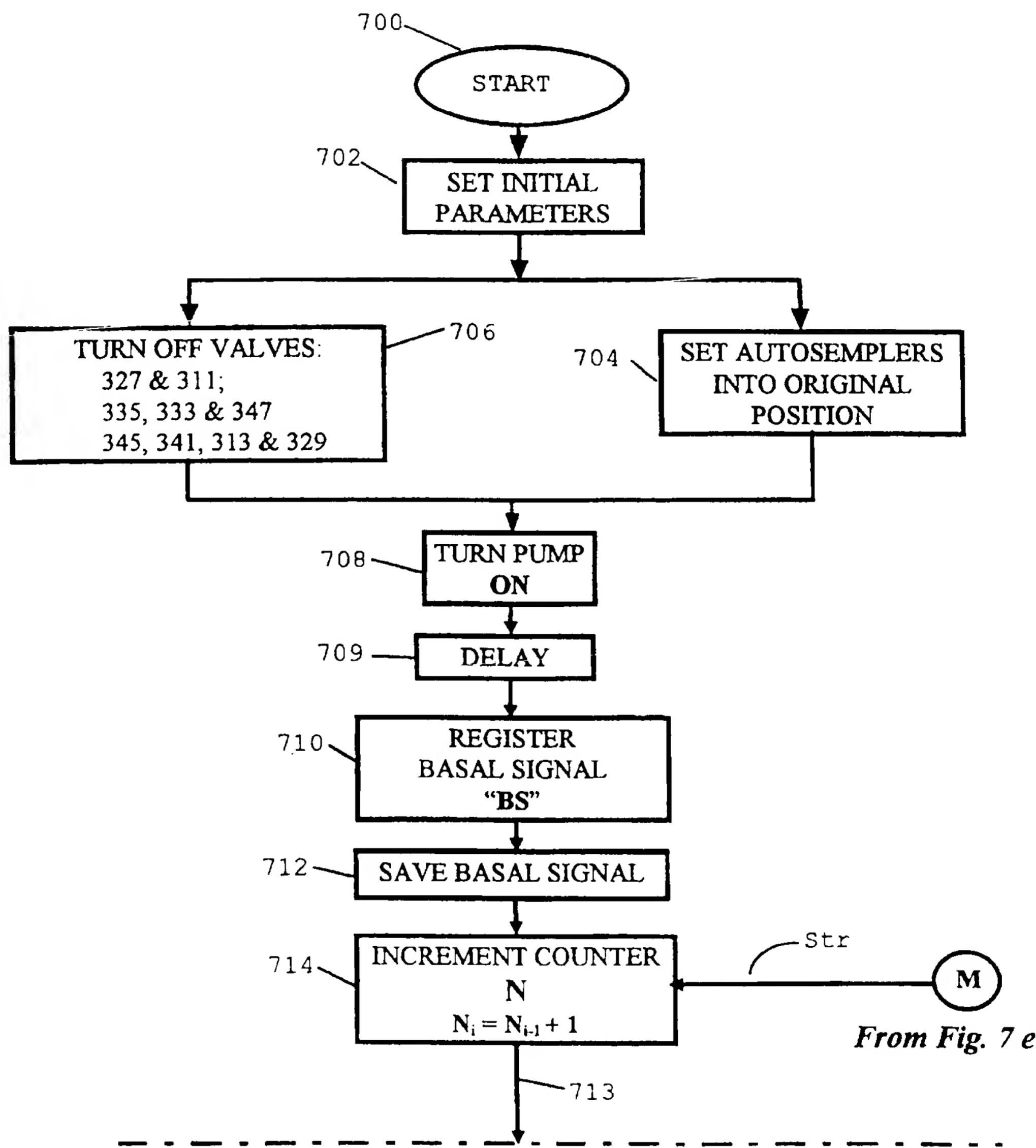
Fig. 6 d

Fig. 7



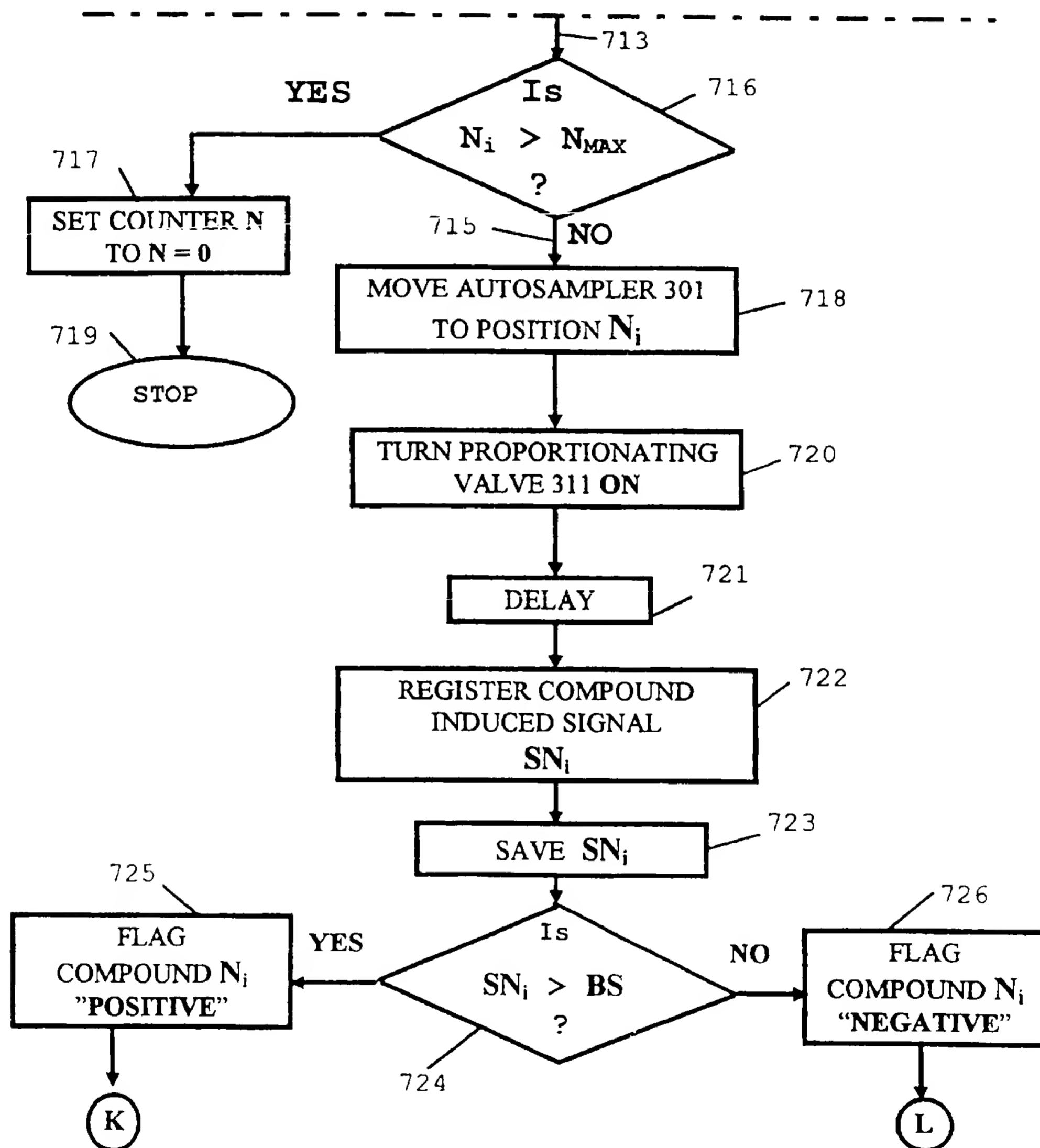
11/42

Fig. 7a



From Fig. 7e

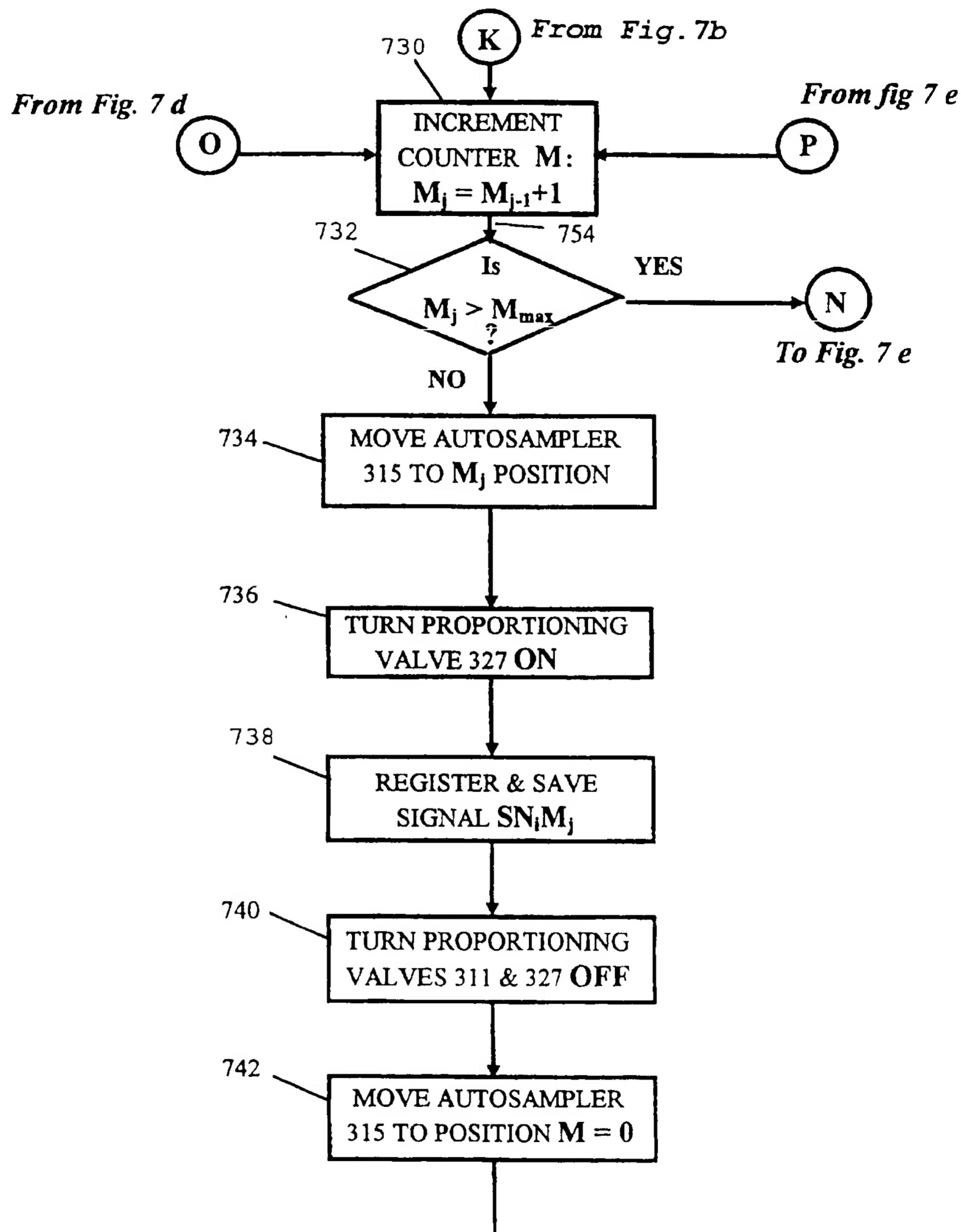
Fig. 7b



To Fig. 7

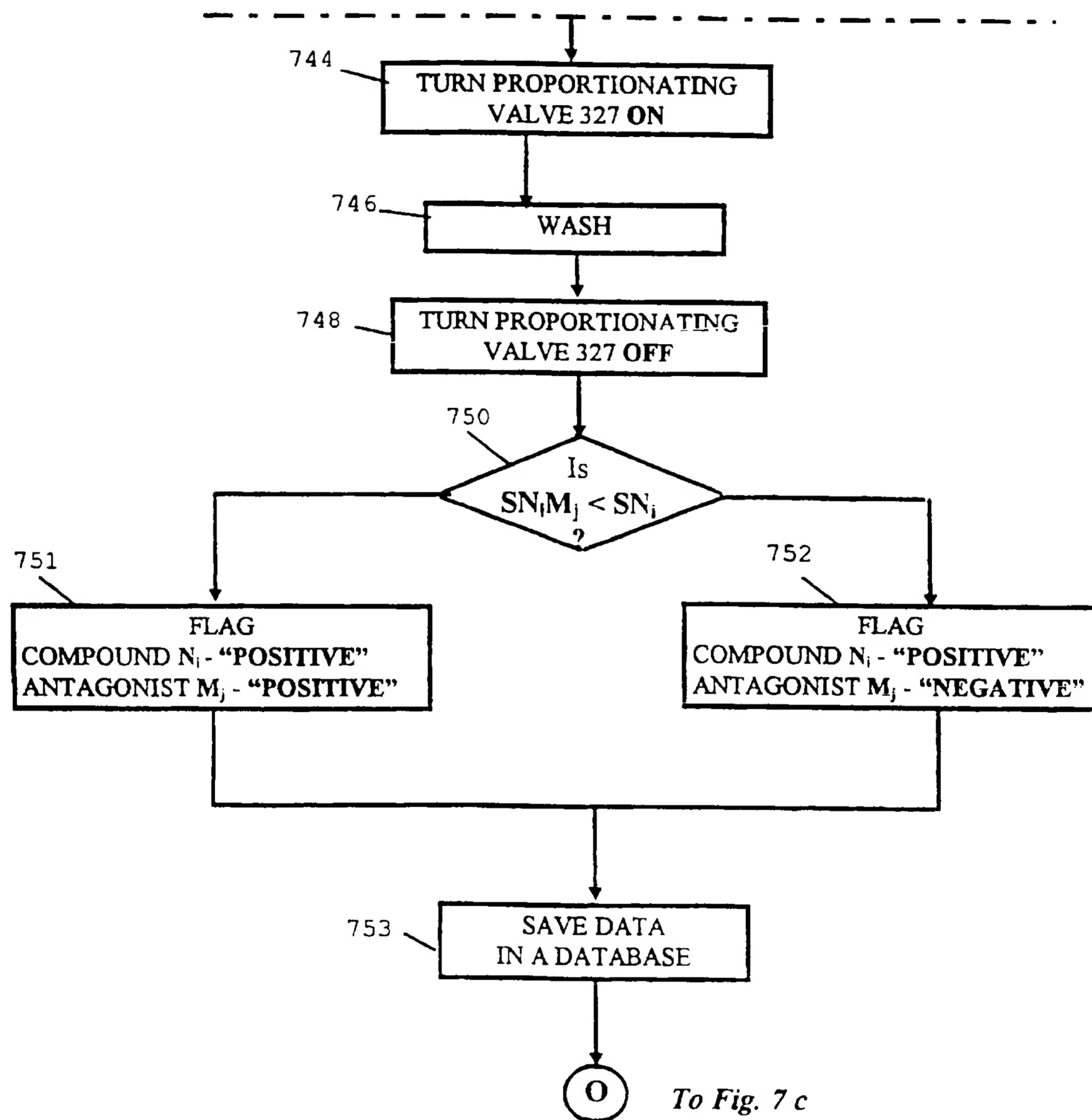
13/4.3

Fig. 7c



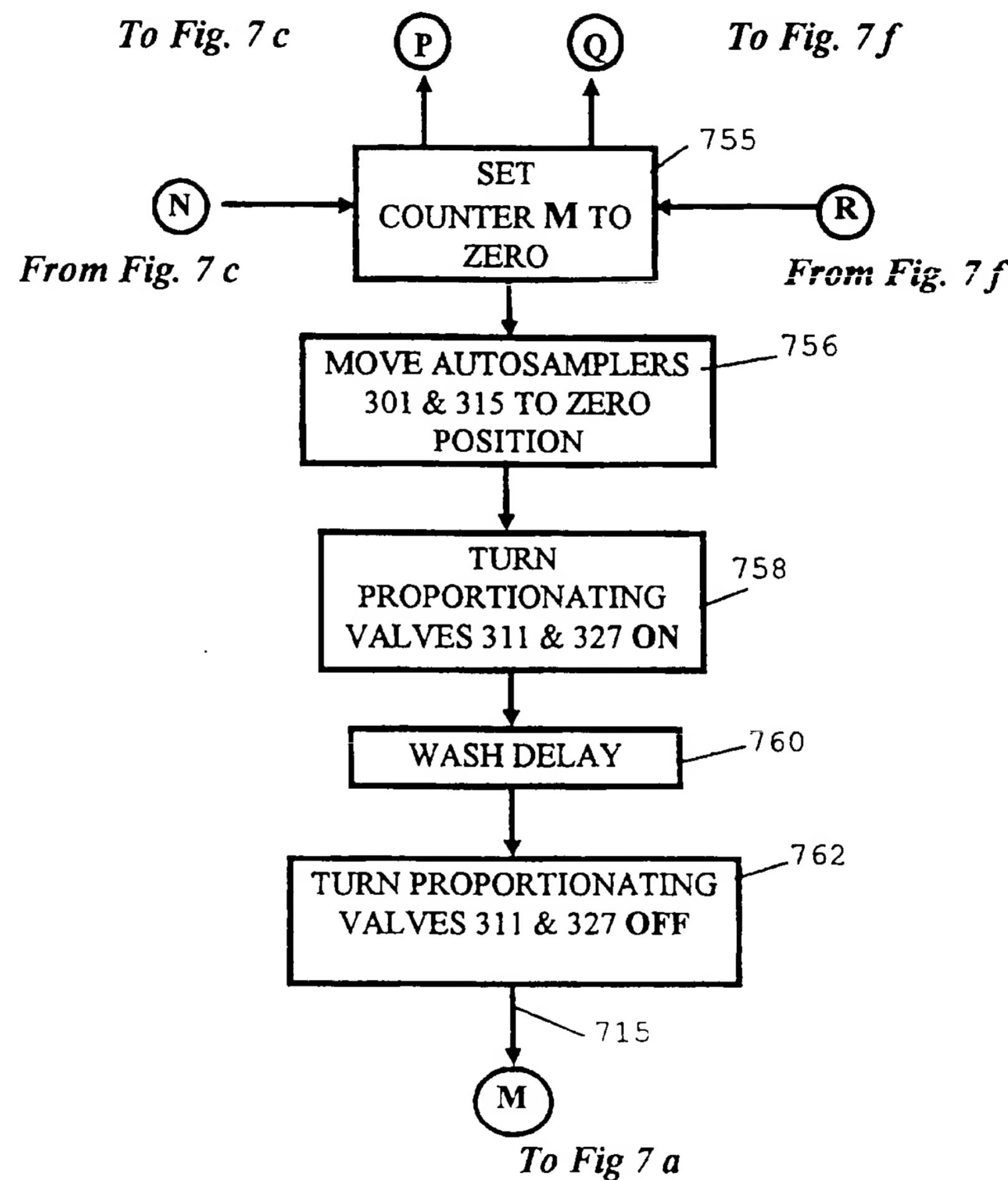
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Fig. 7d



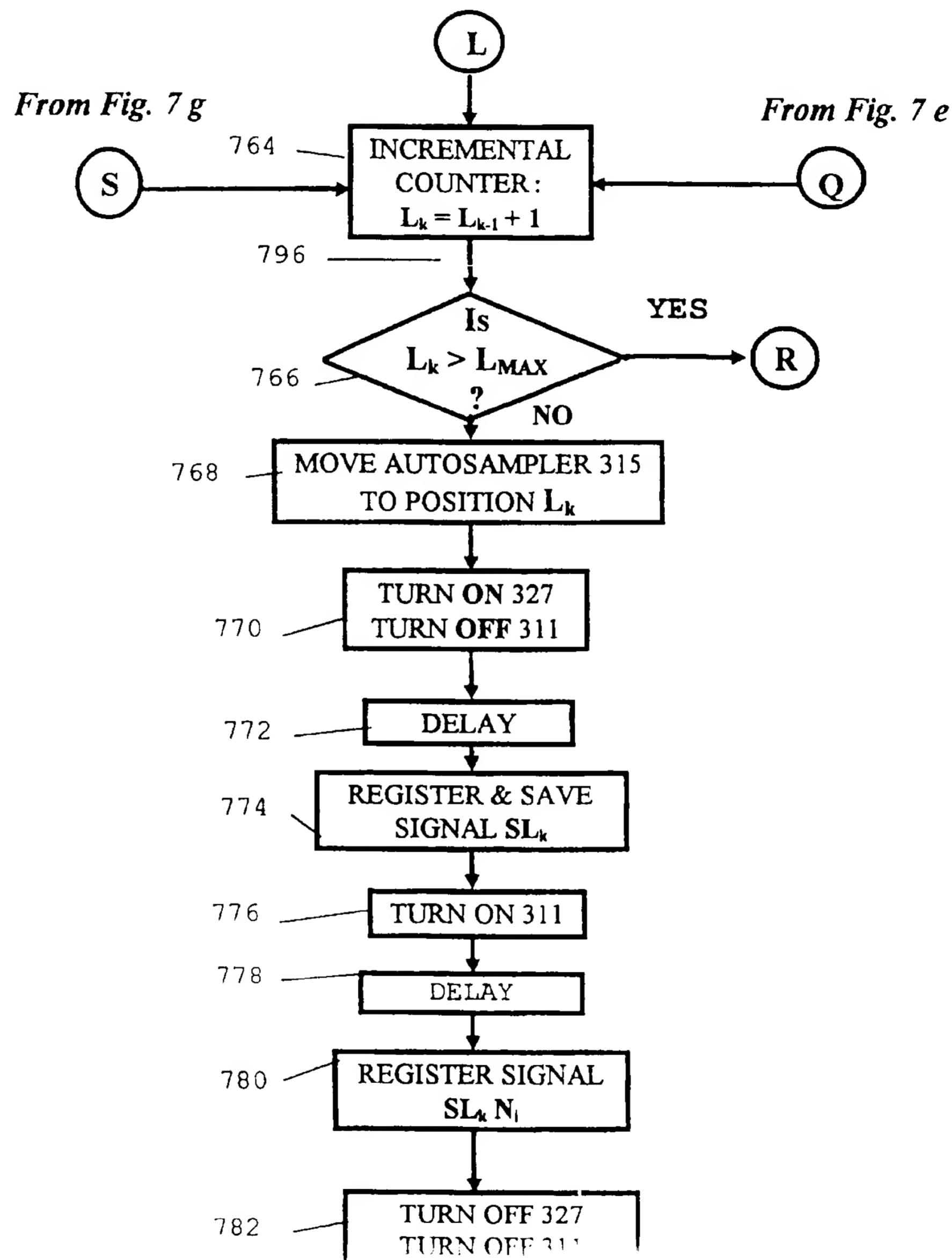
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Fig. 7 e



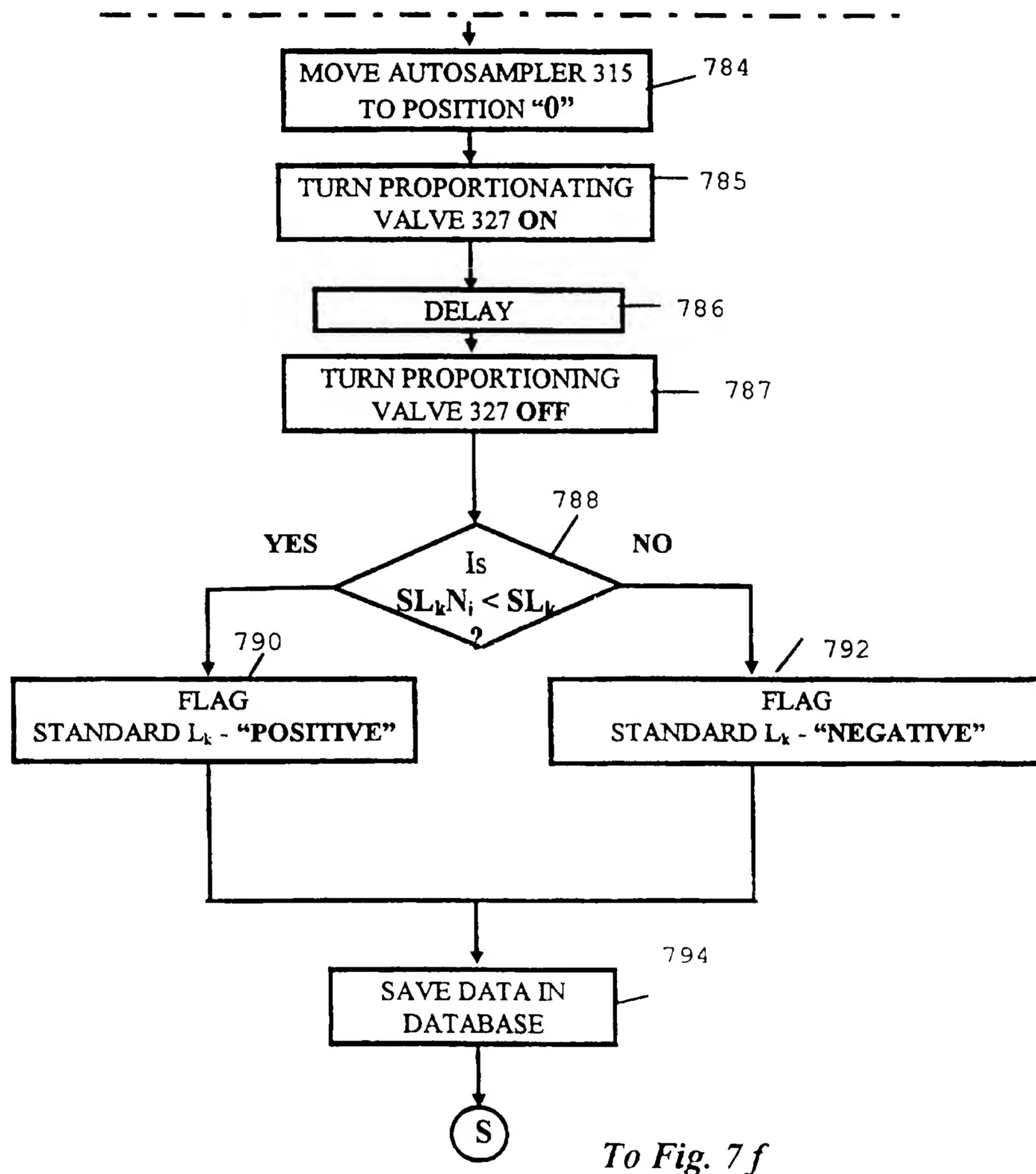
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Fig. 7 f



10/10

Fig. 7 g



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Fig. 8

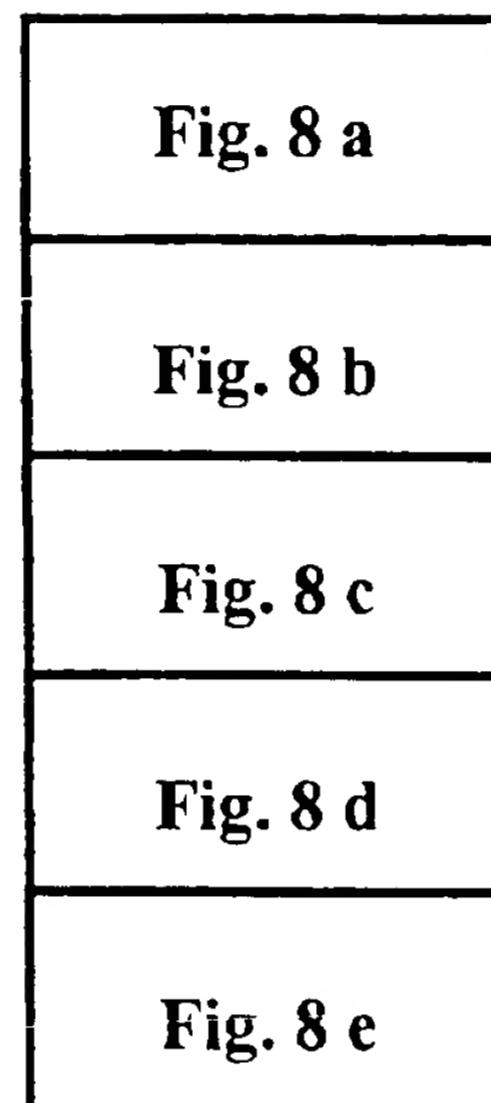
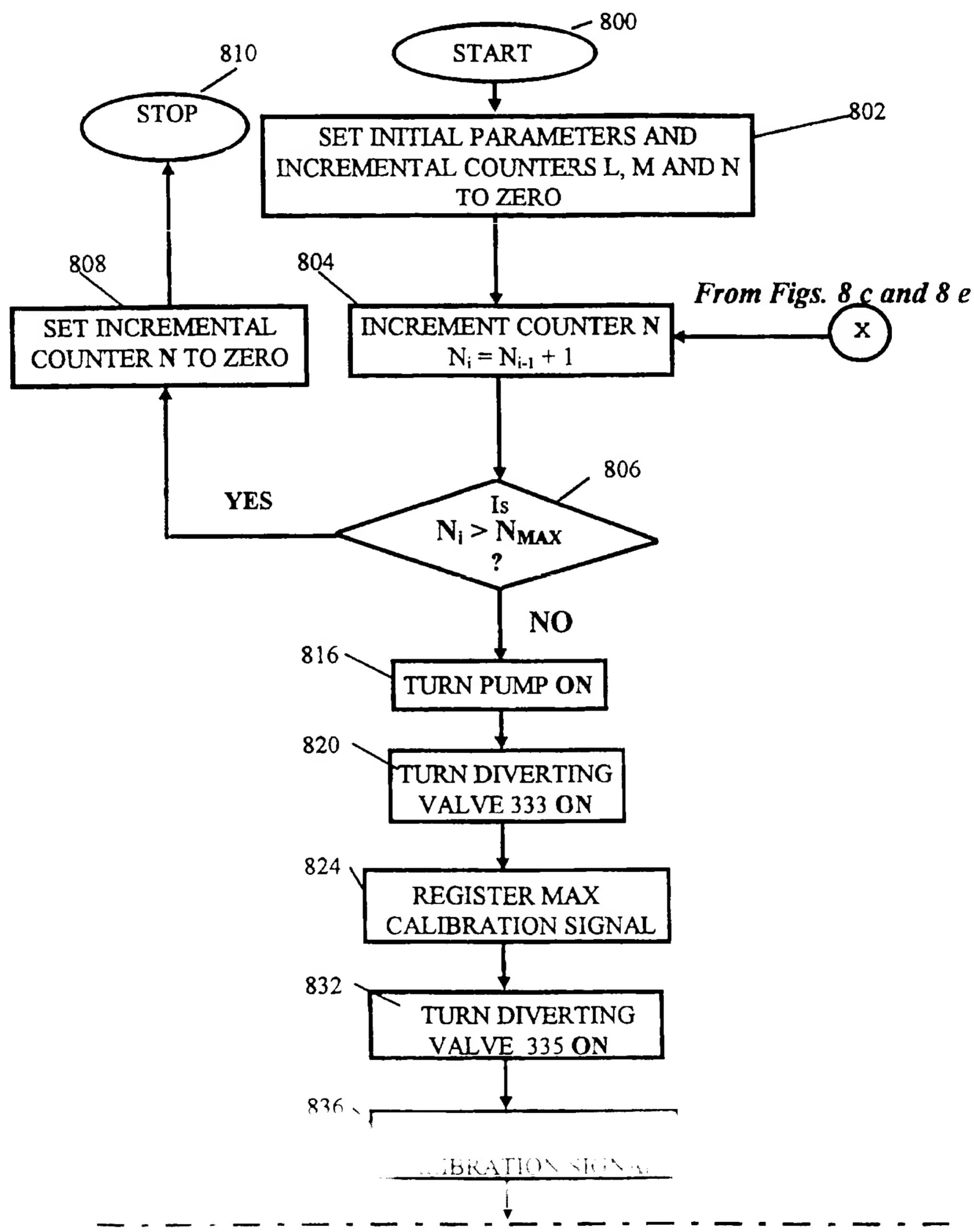
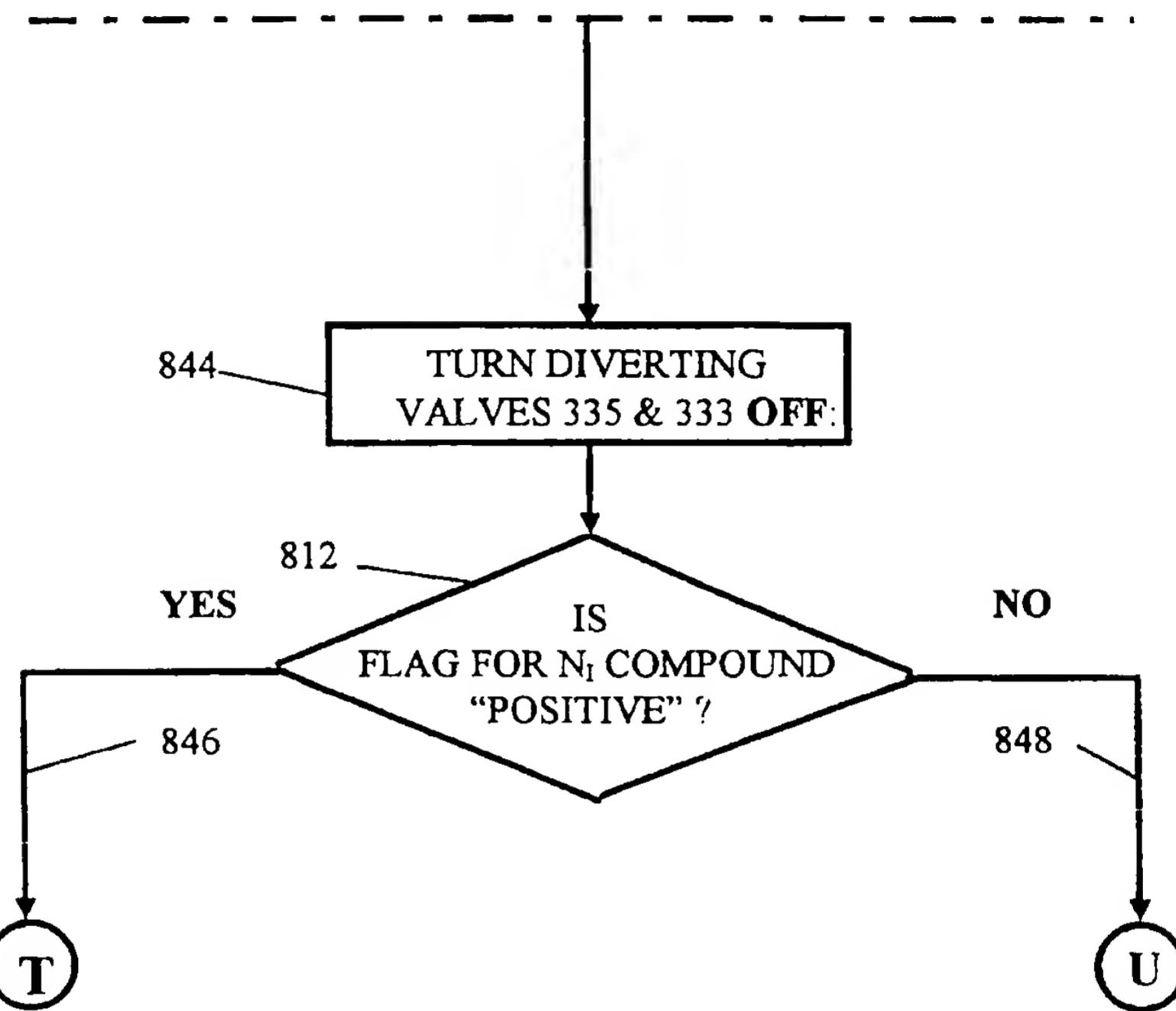


Fig. 8 a



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Fig. 8 b*To Fig. 8 c**To Fig. 8 e*

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Fig. 8 c

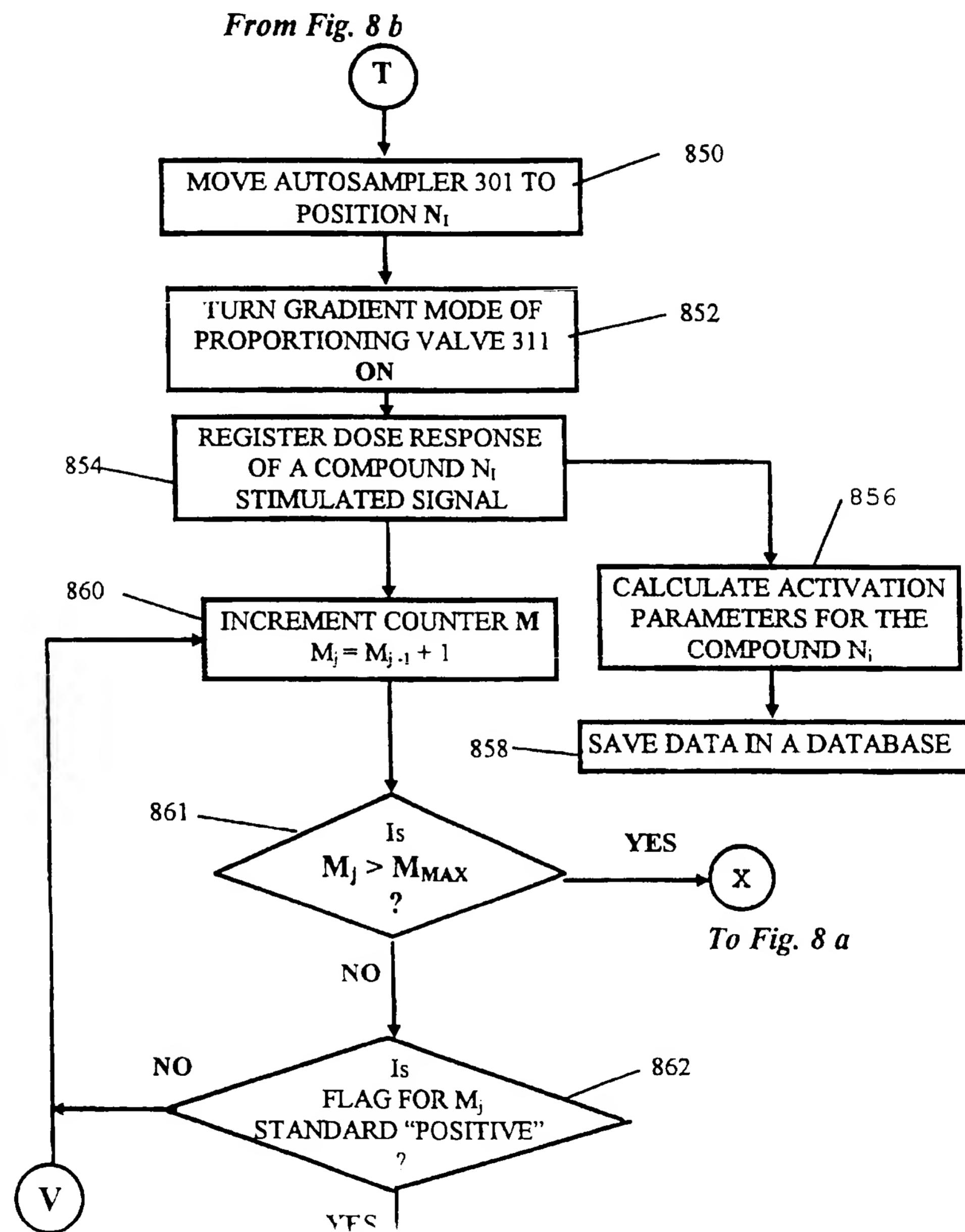
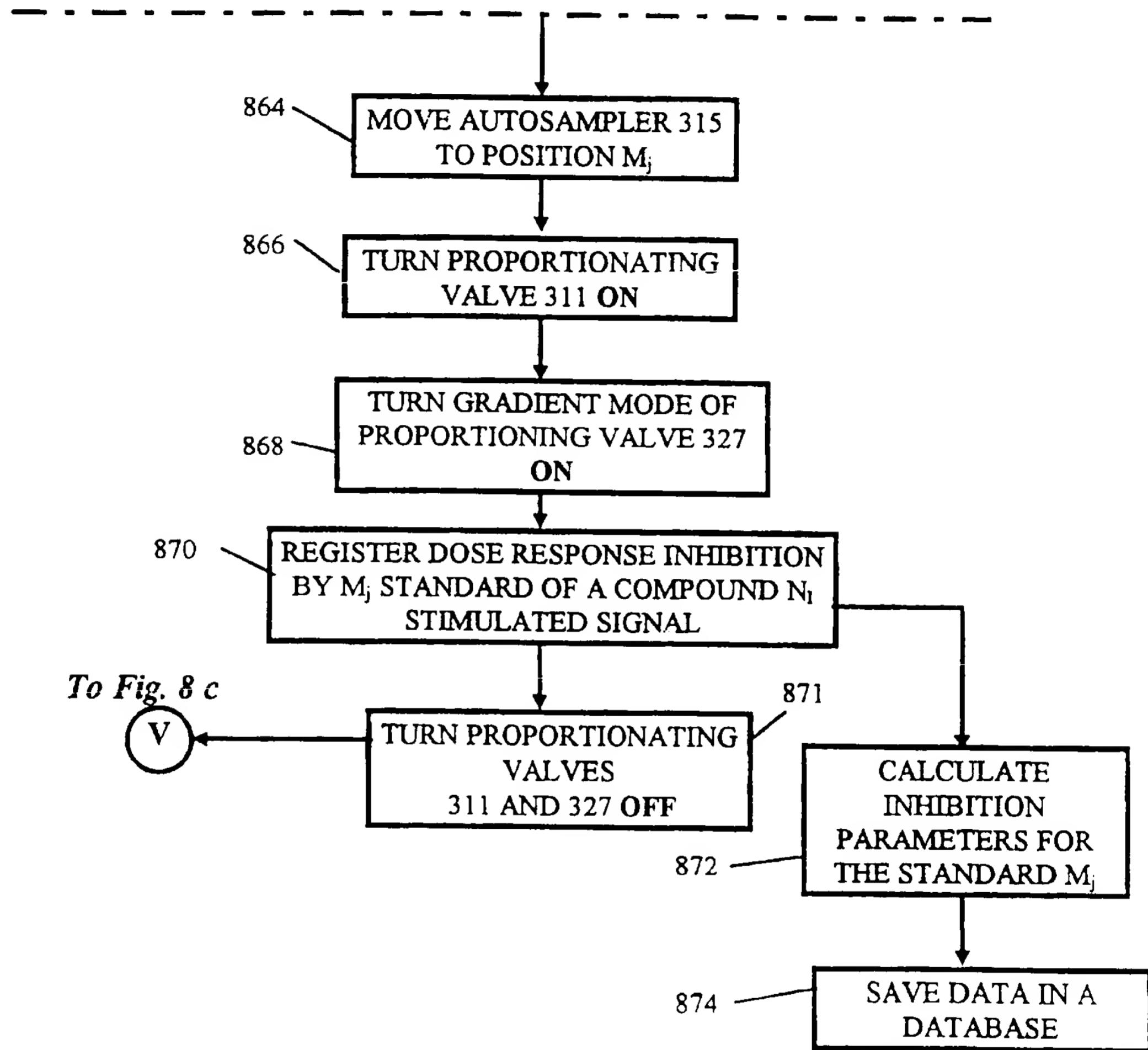


Fig. 8 d



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Fig. 8 e

From Fig. 8 b

To Fig. 8a

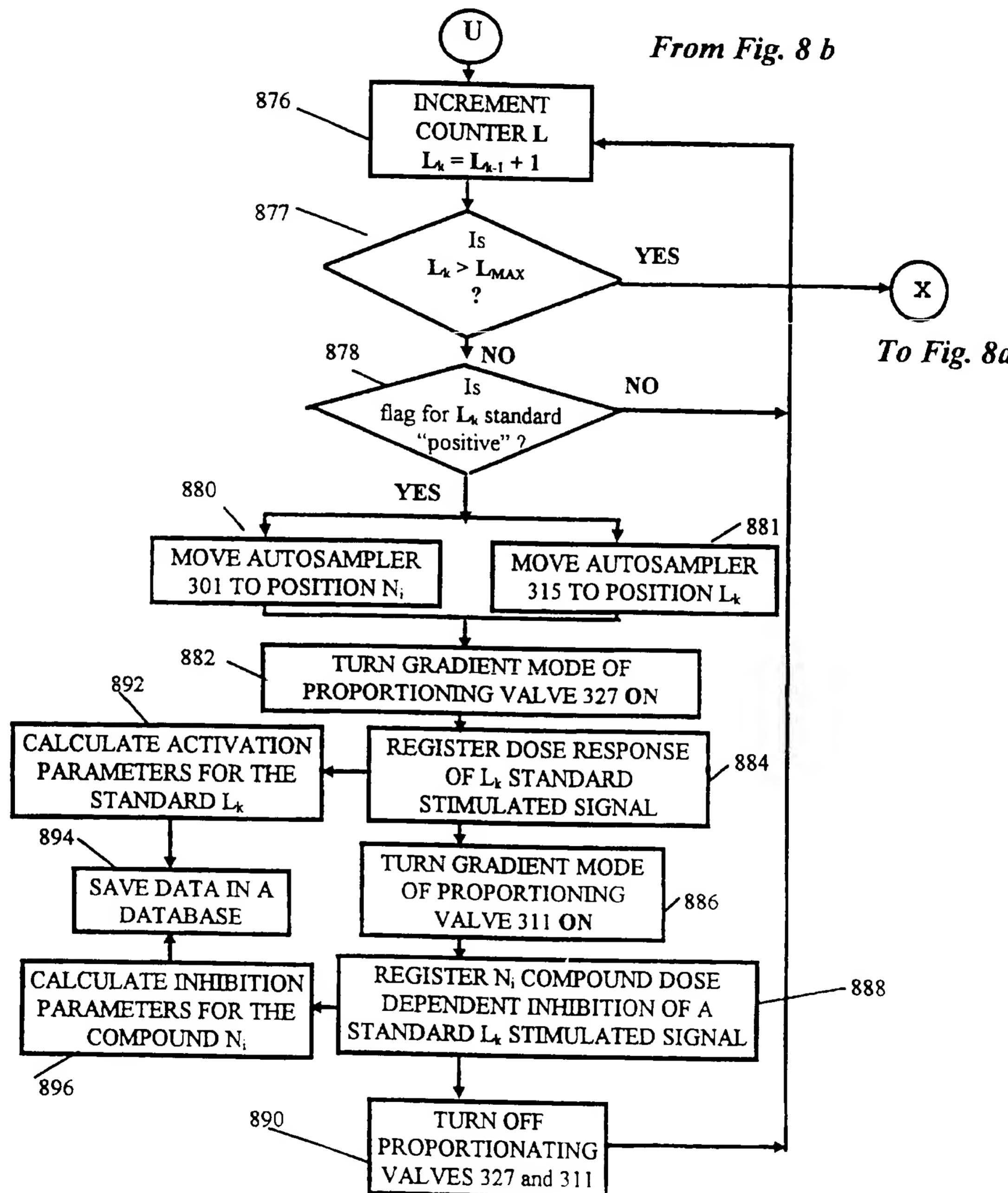


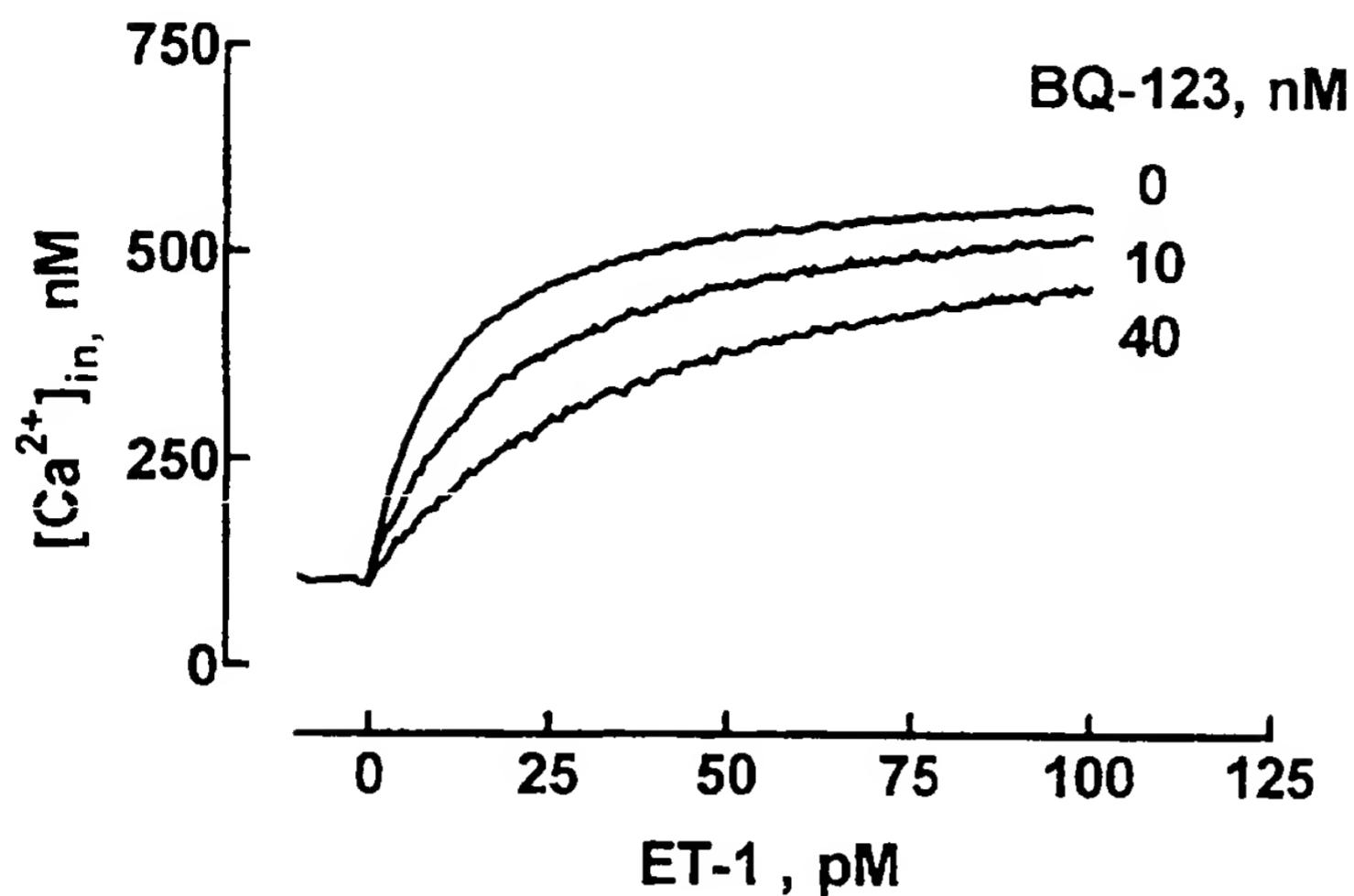
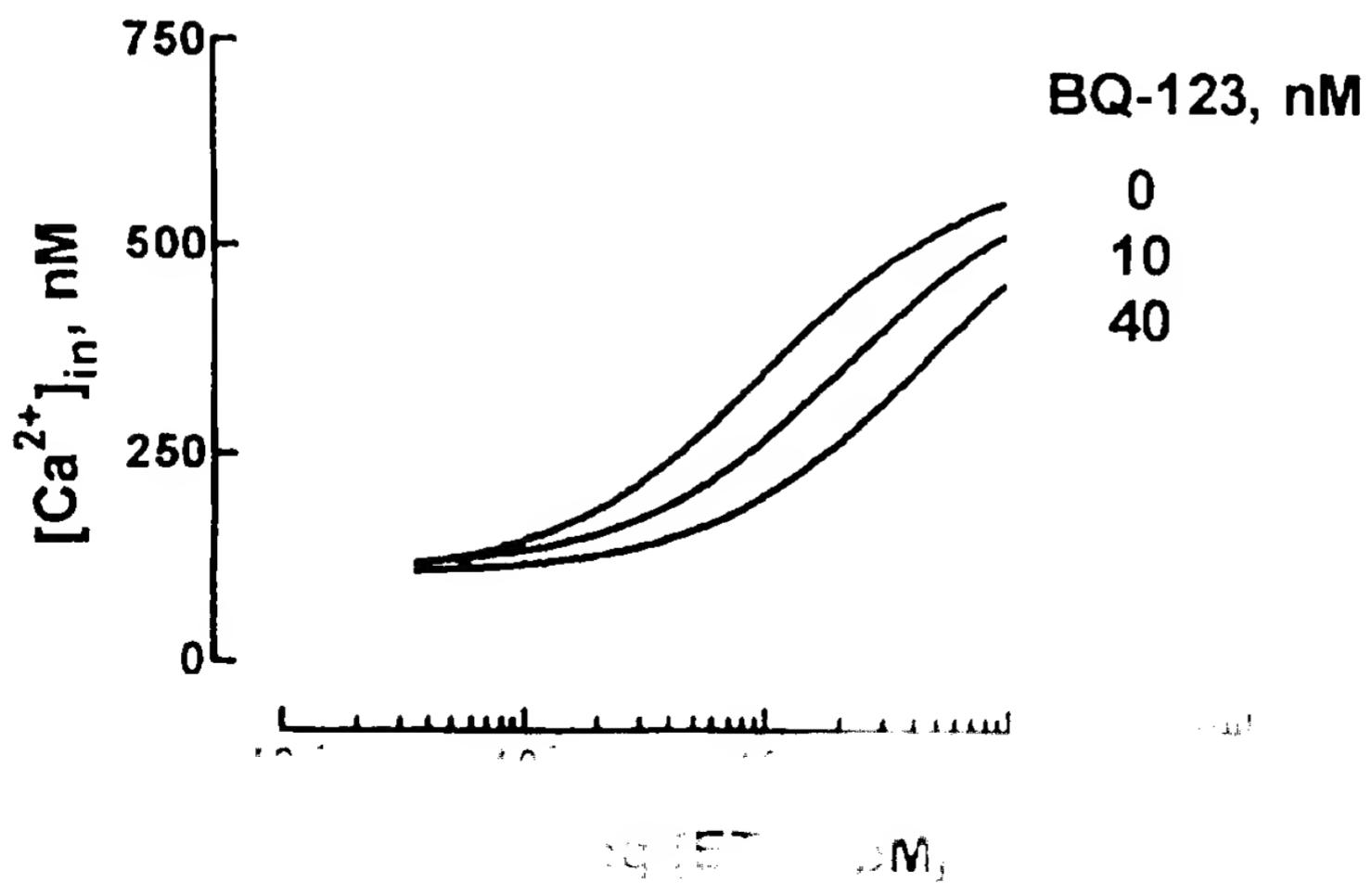
Fig. 9**Fig. 10**

Fig. 11

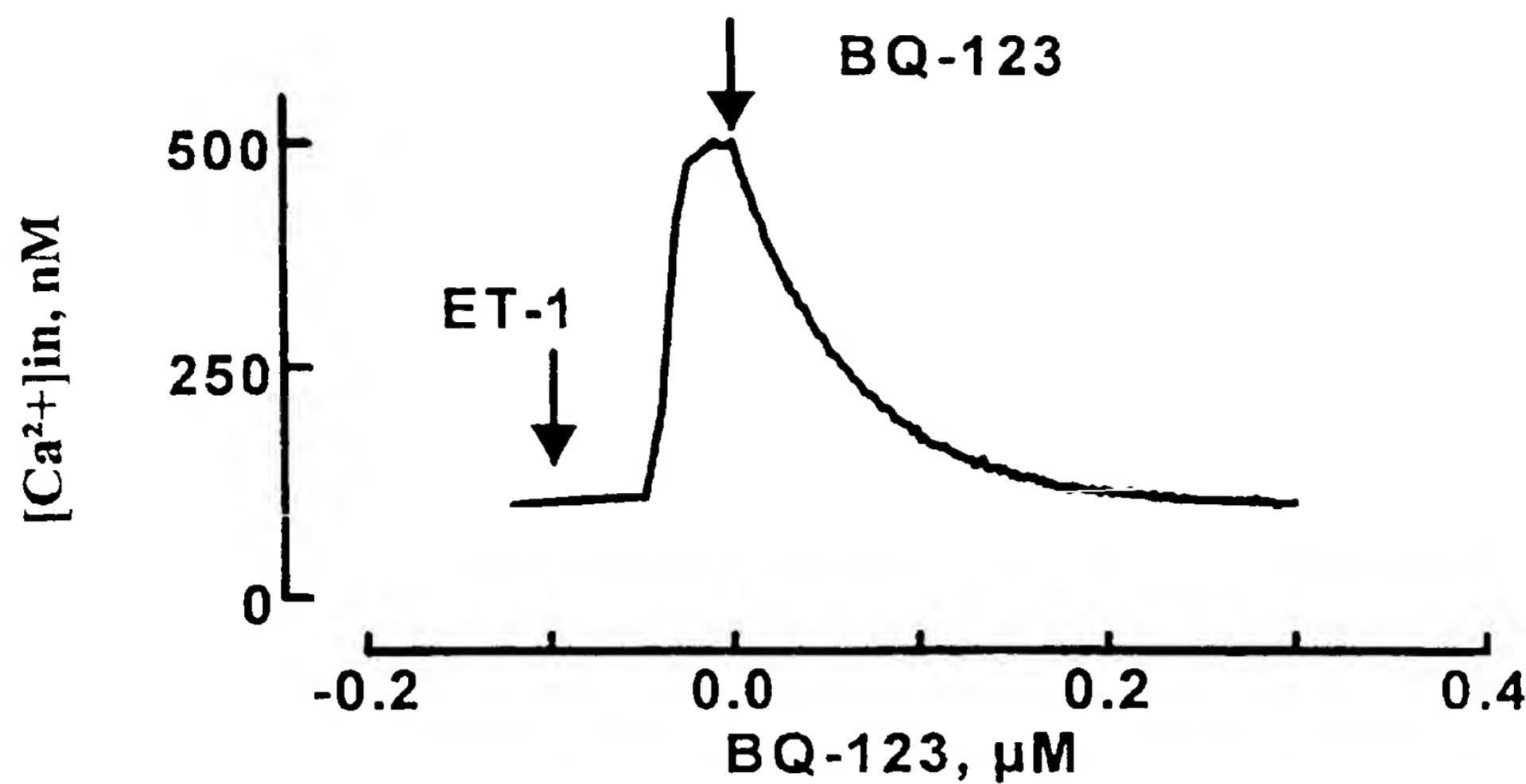
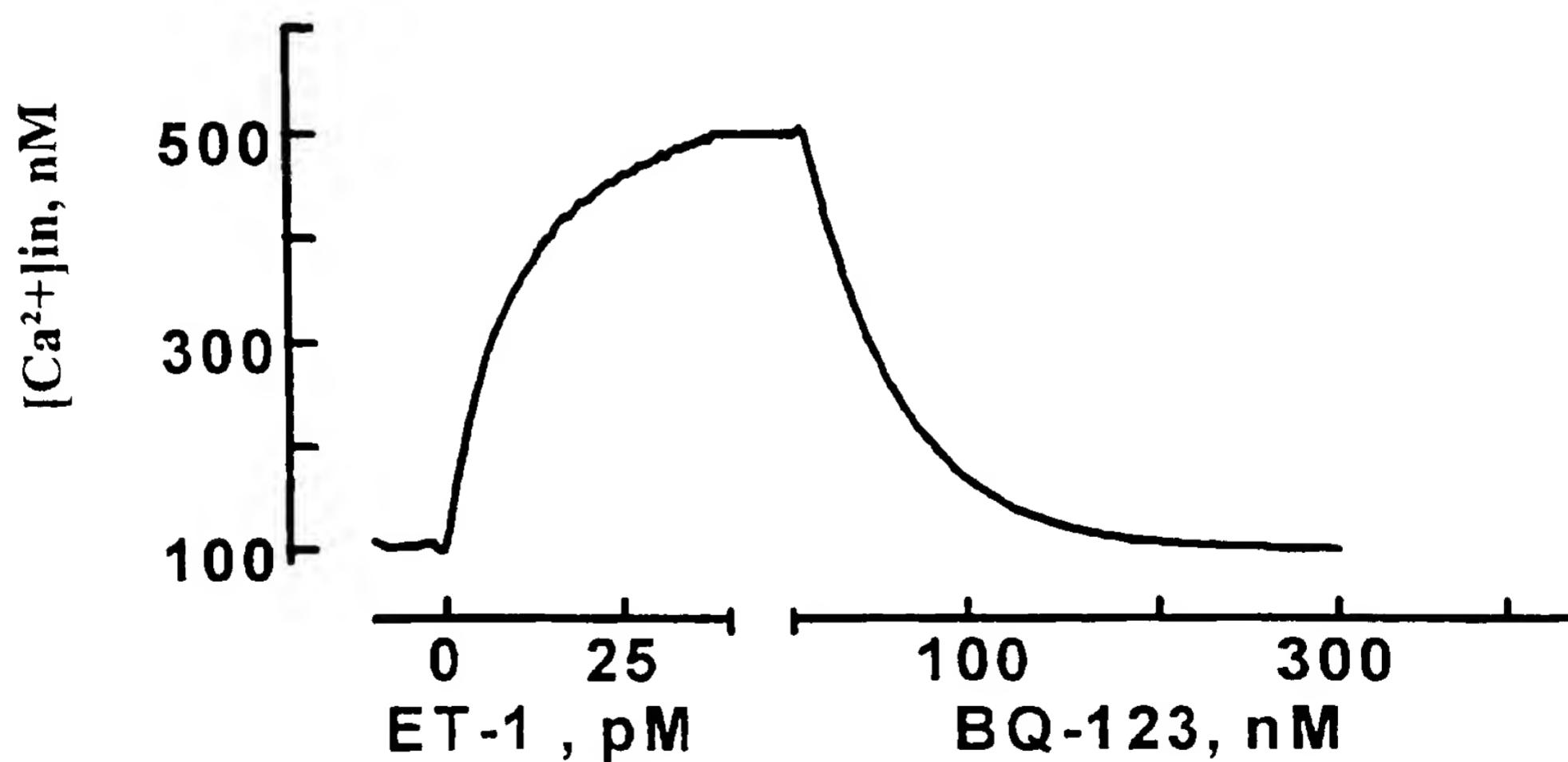


Fig. 12



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Fig. 13

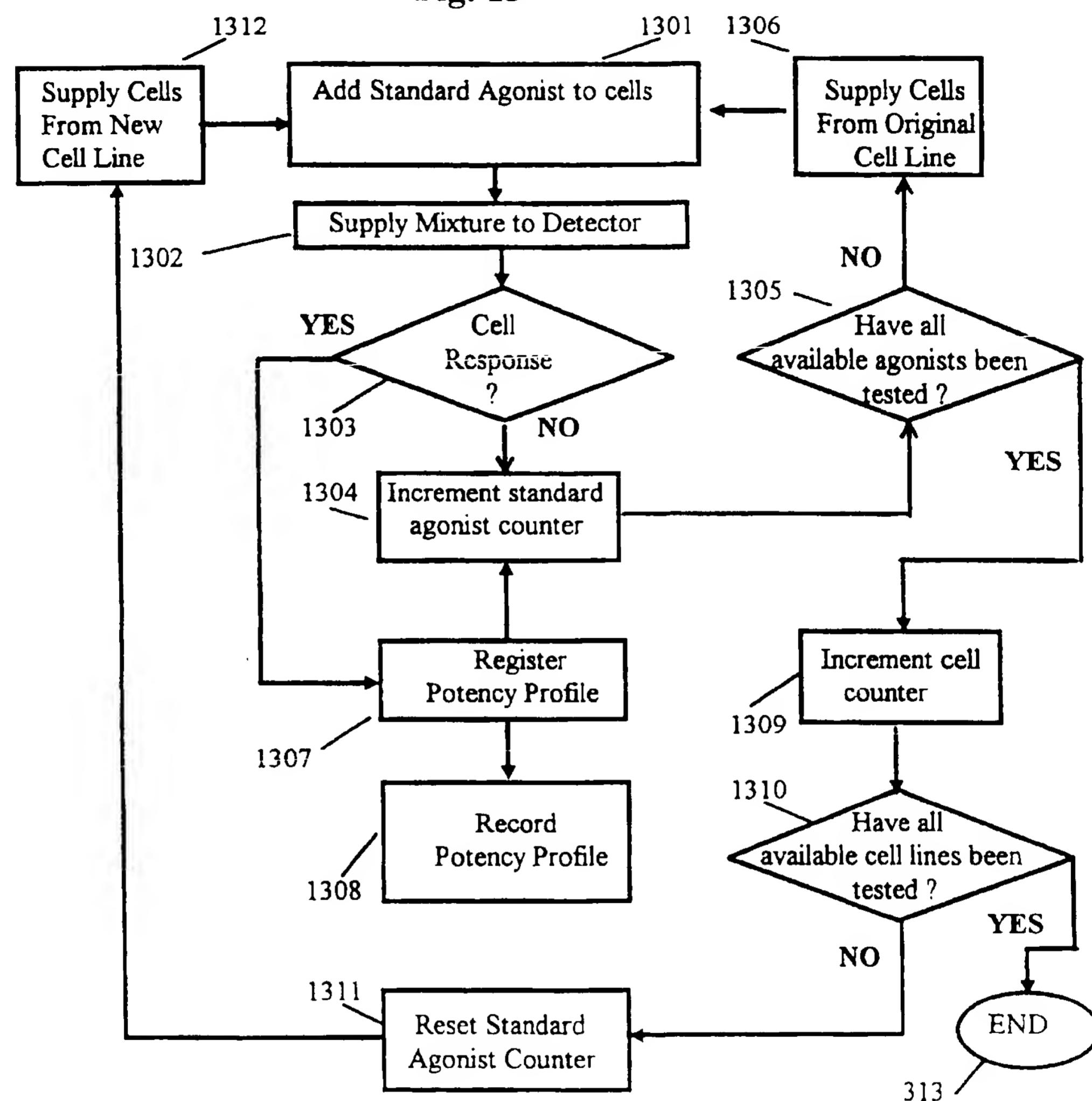
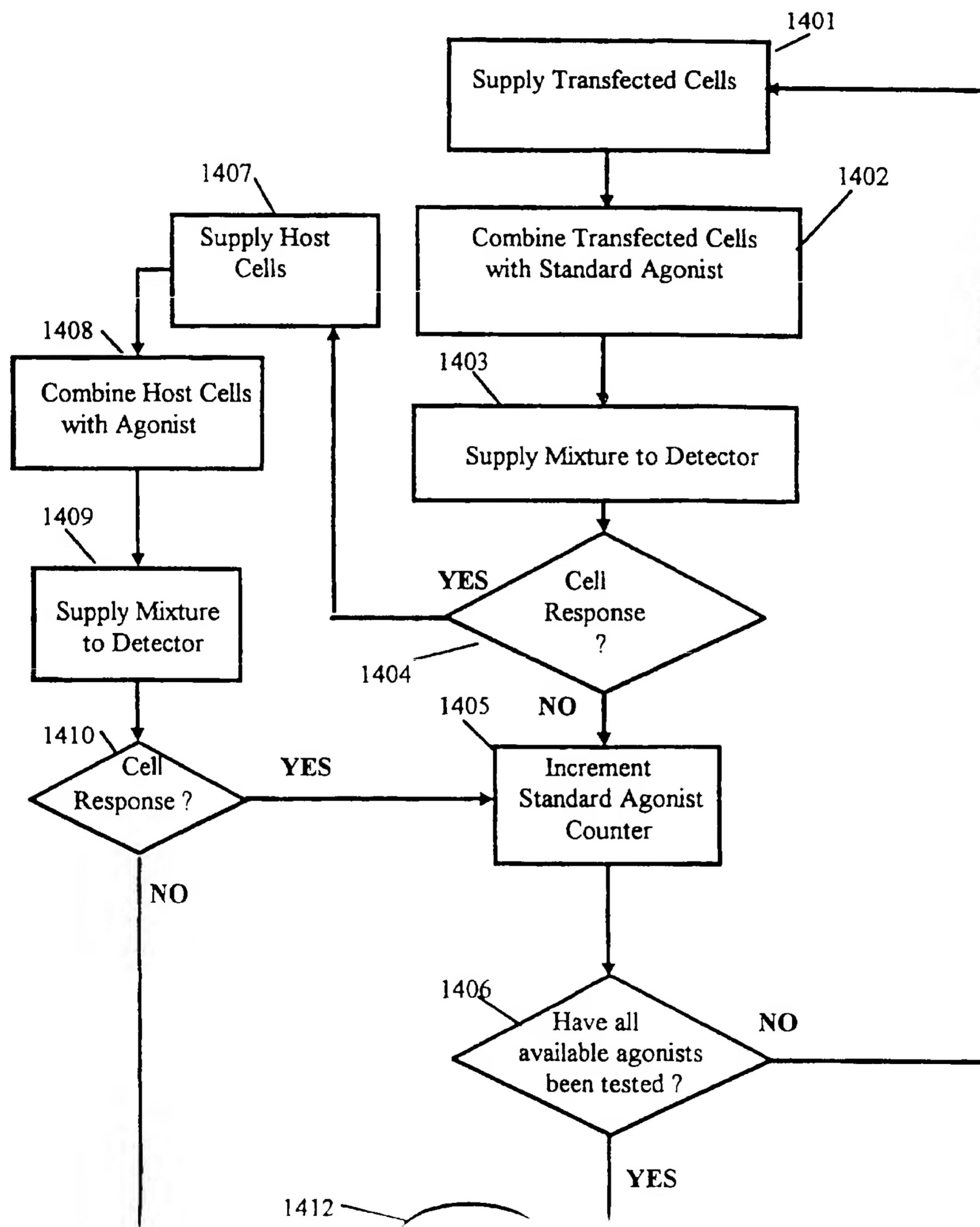
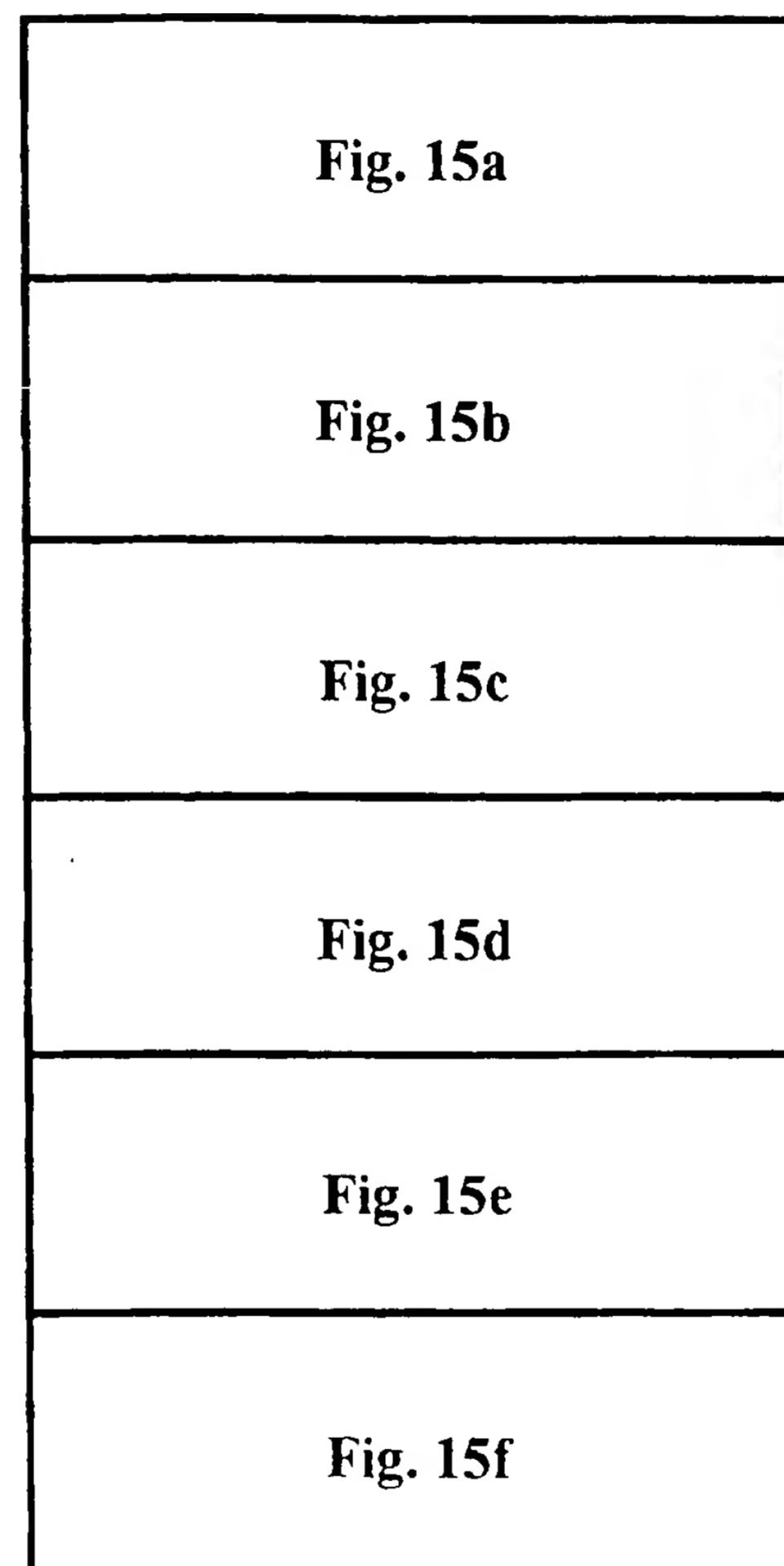


Fig. 14



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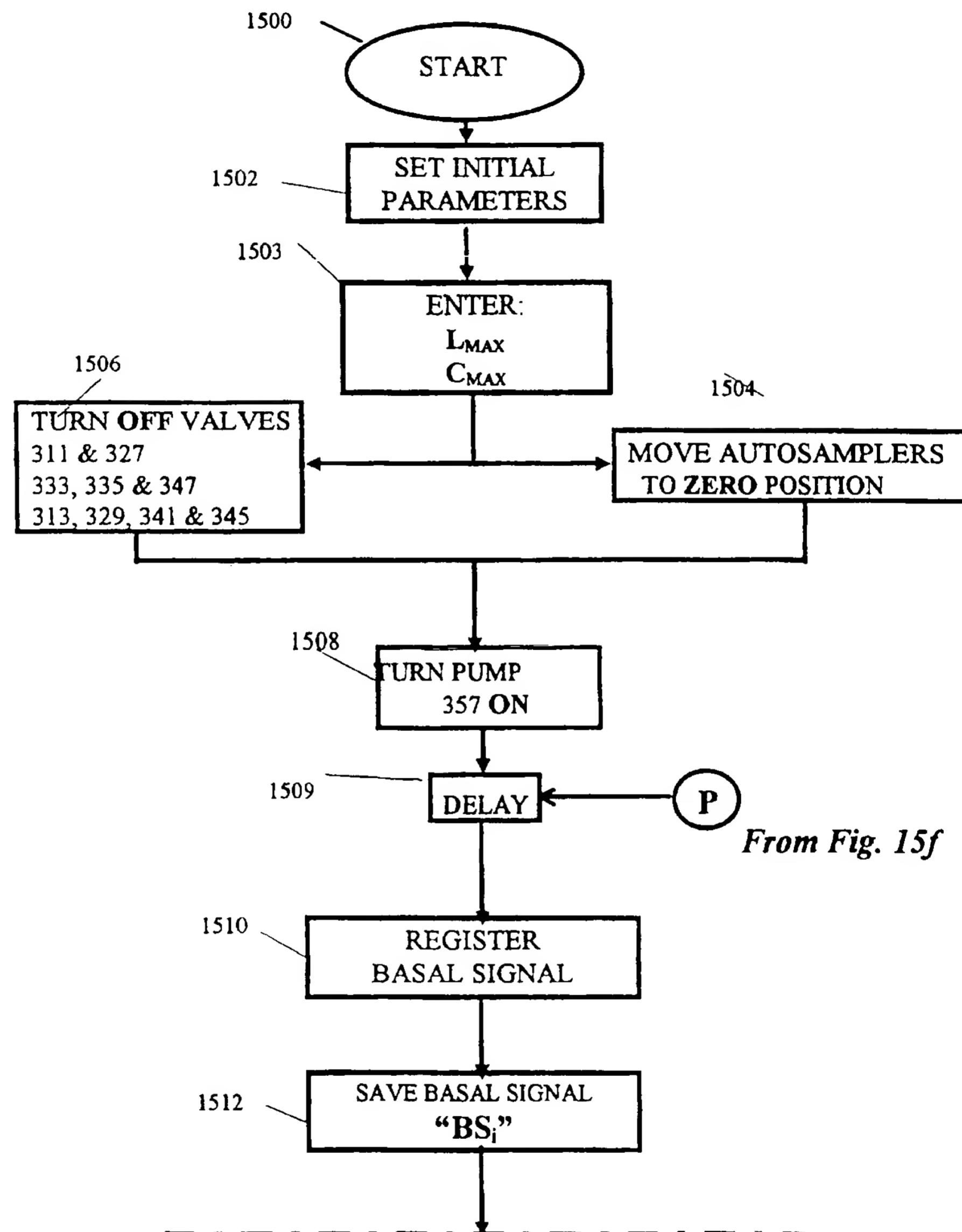
Fig. 15



29/4/02

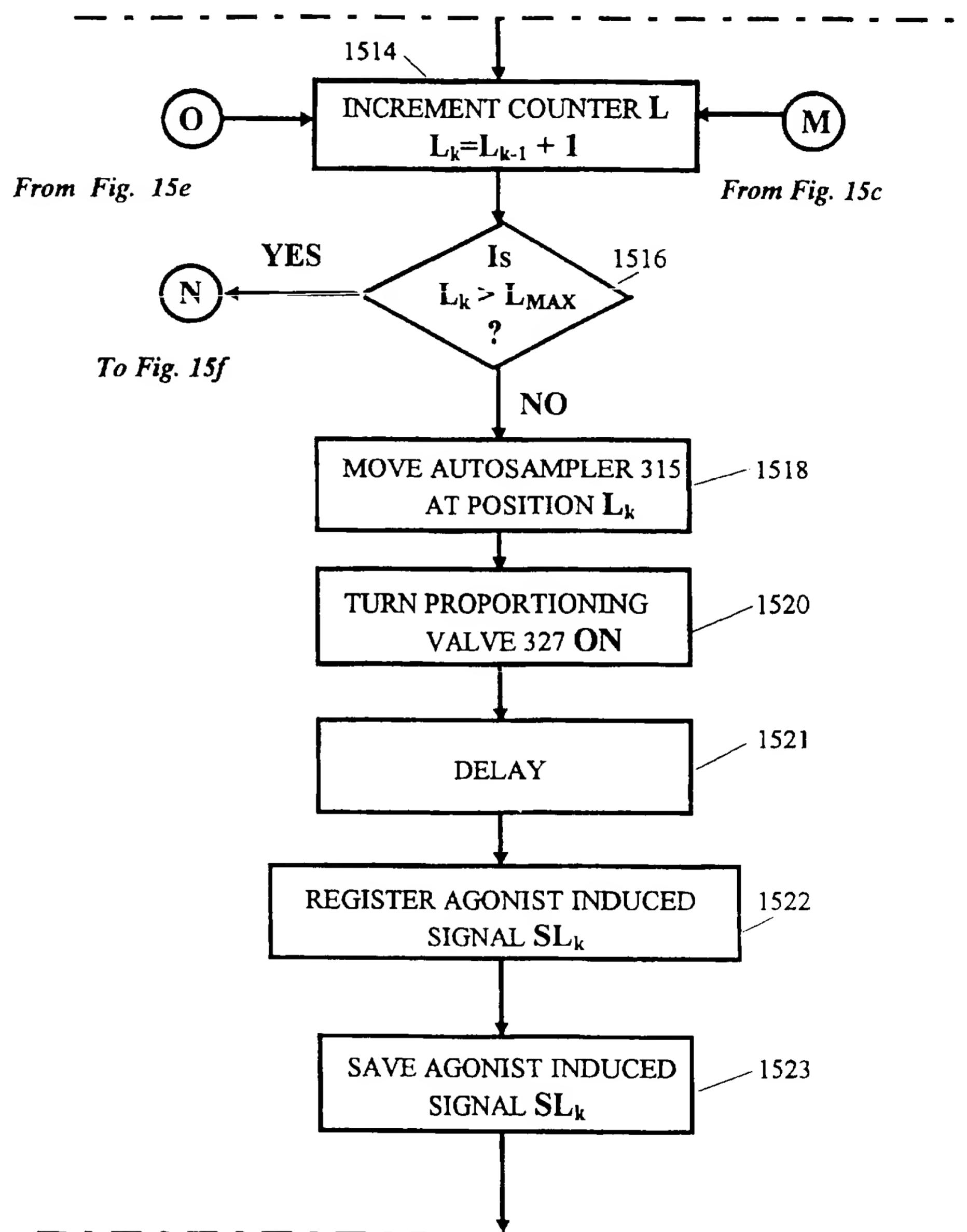
13

Fig. 15a



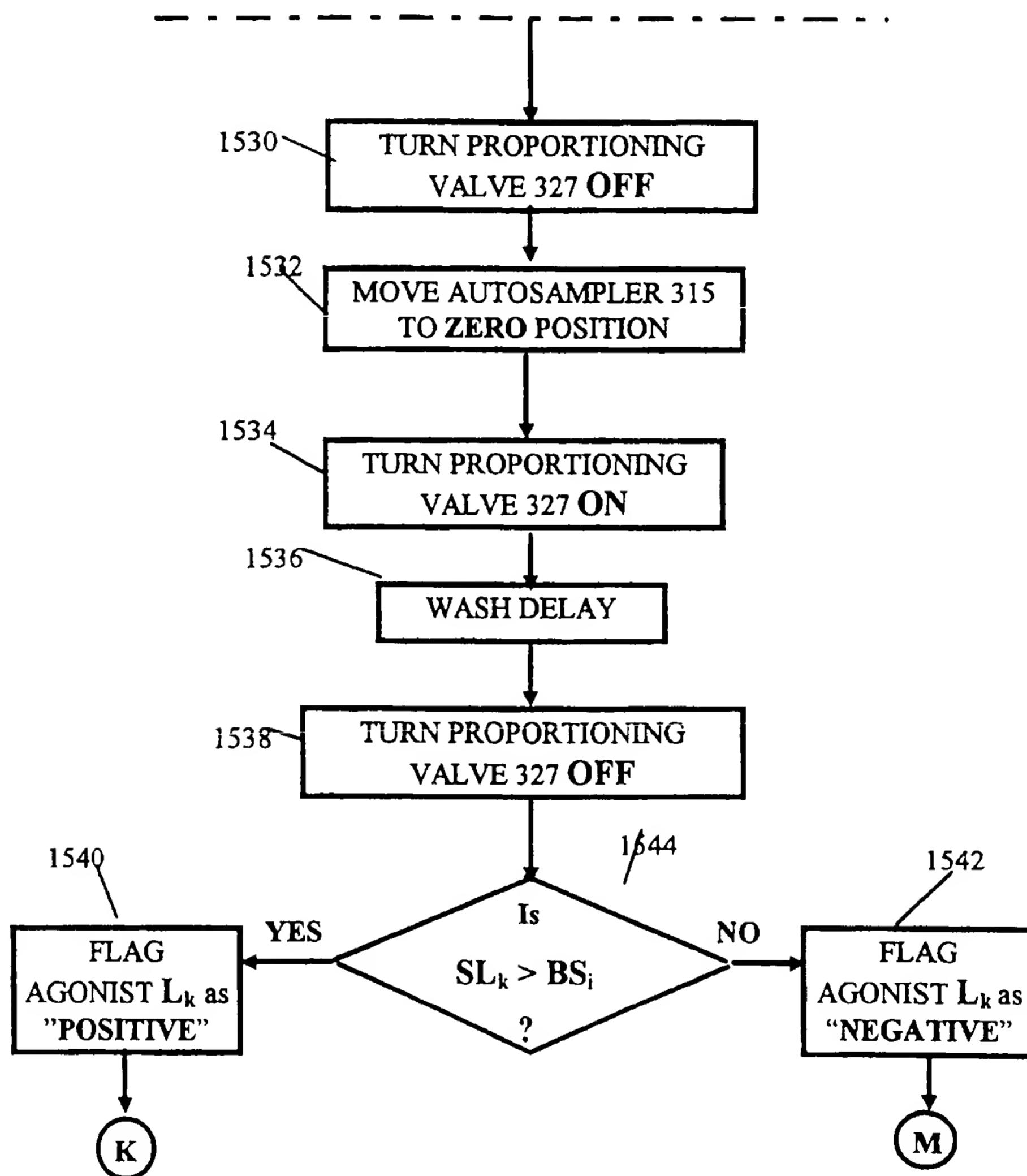
From Fig. 15f

Fig. 15b



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Fig. 15c



To Fig. 15d

To Fig. 15b

Fig. 15d

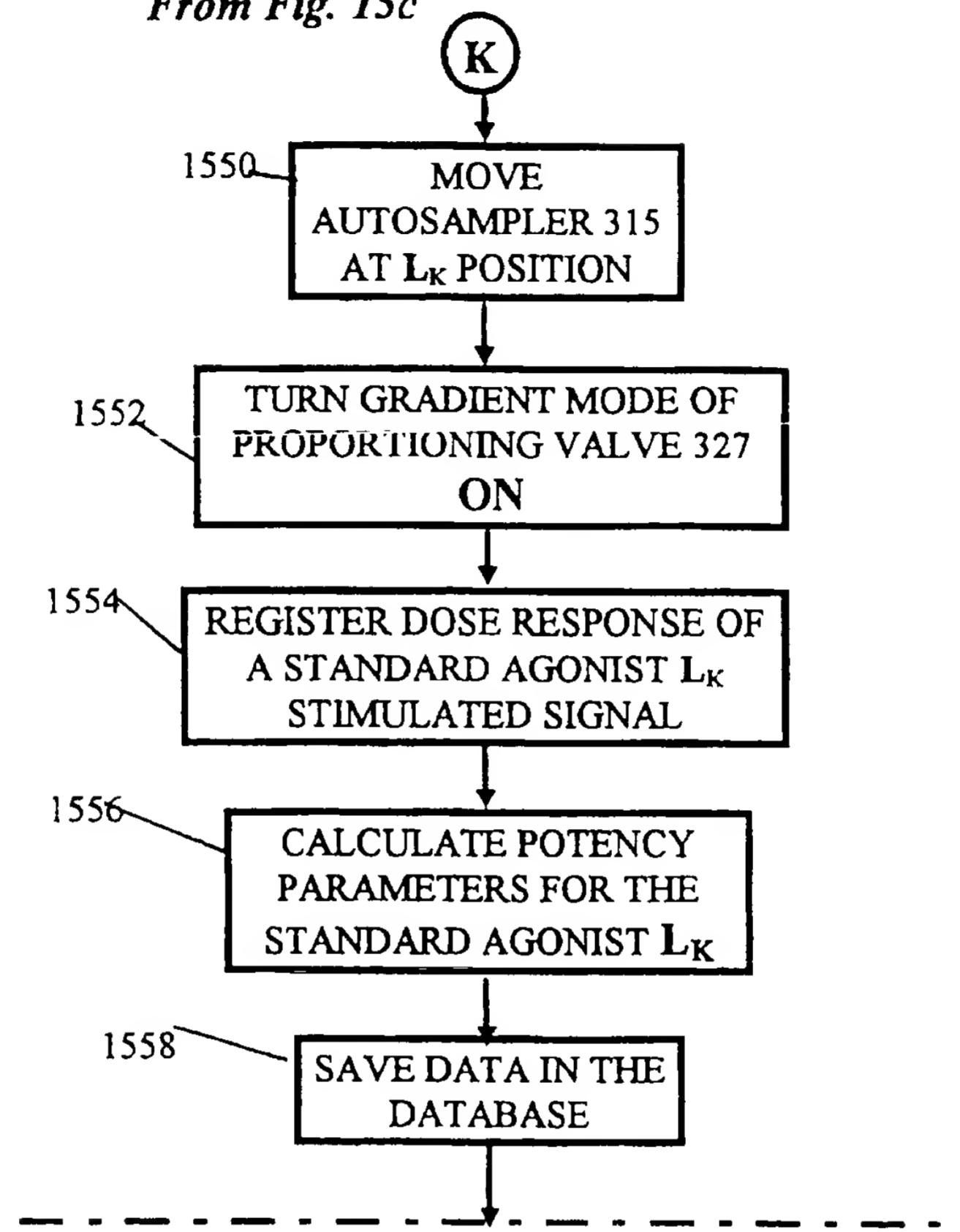
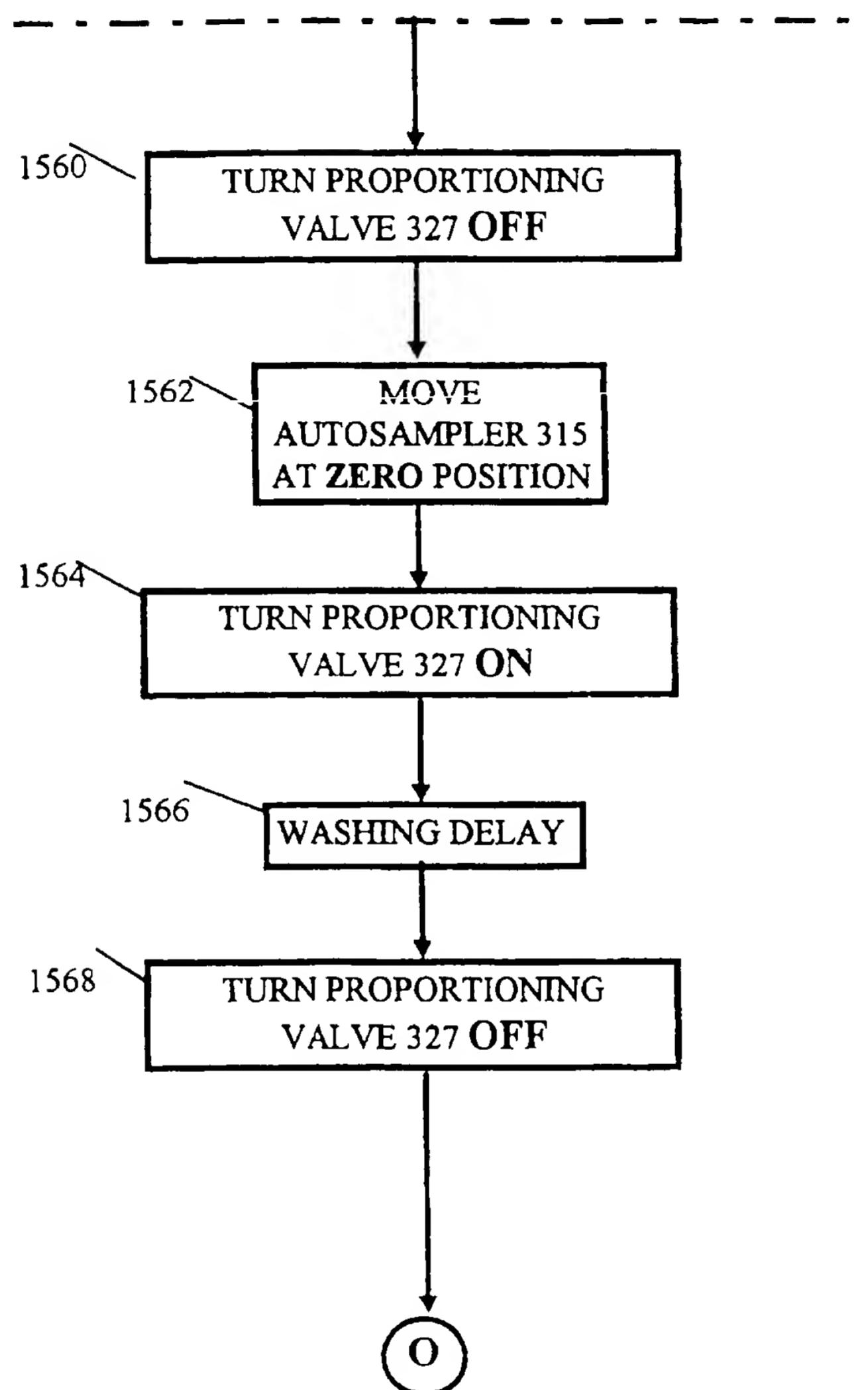
From Fig. 15c

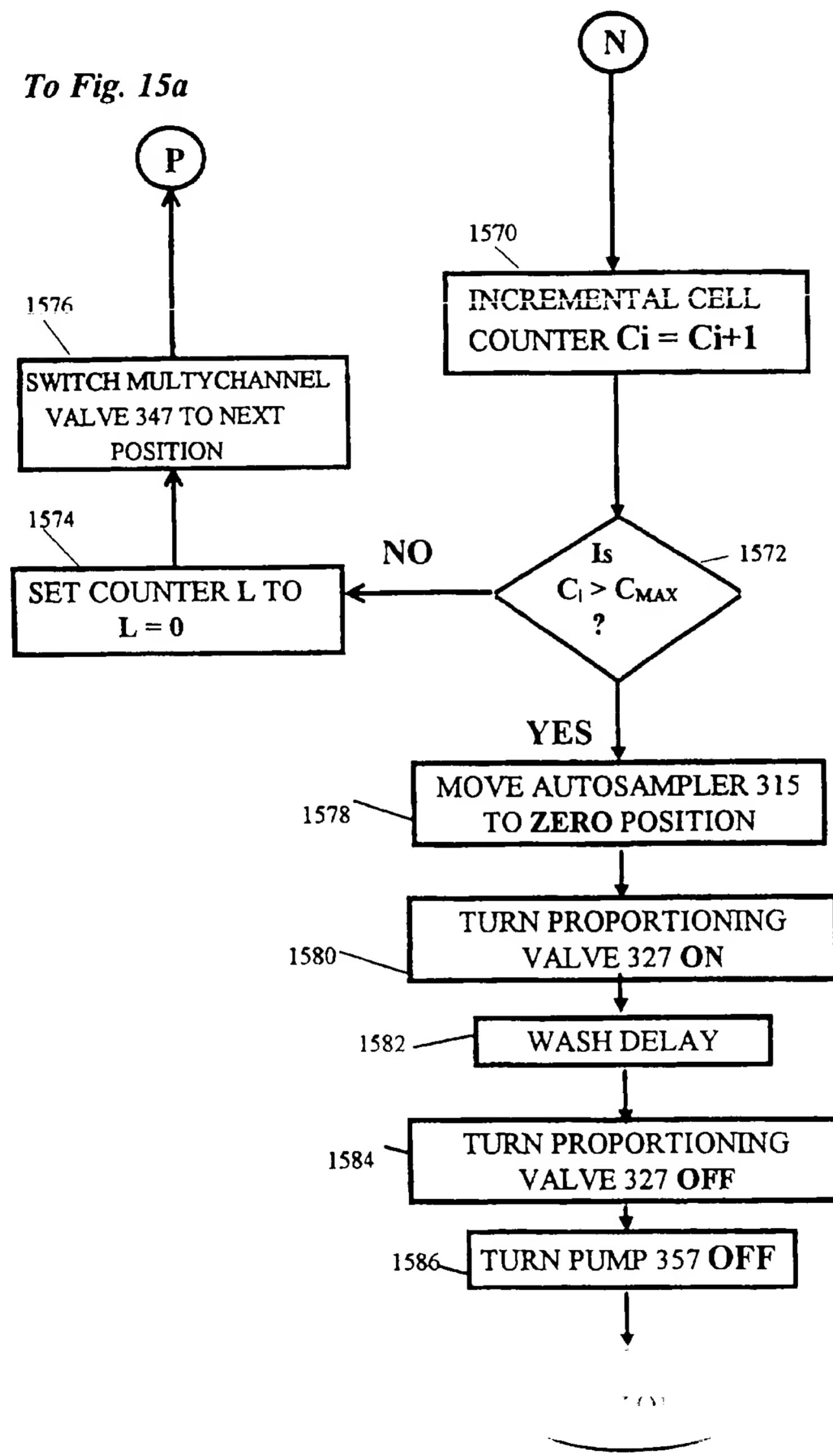
Fig. 15e



To Fig. 15b

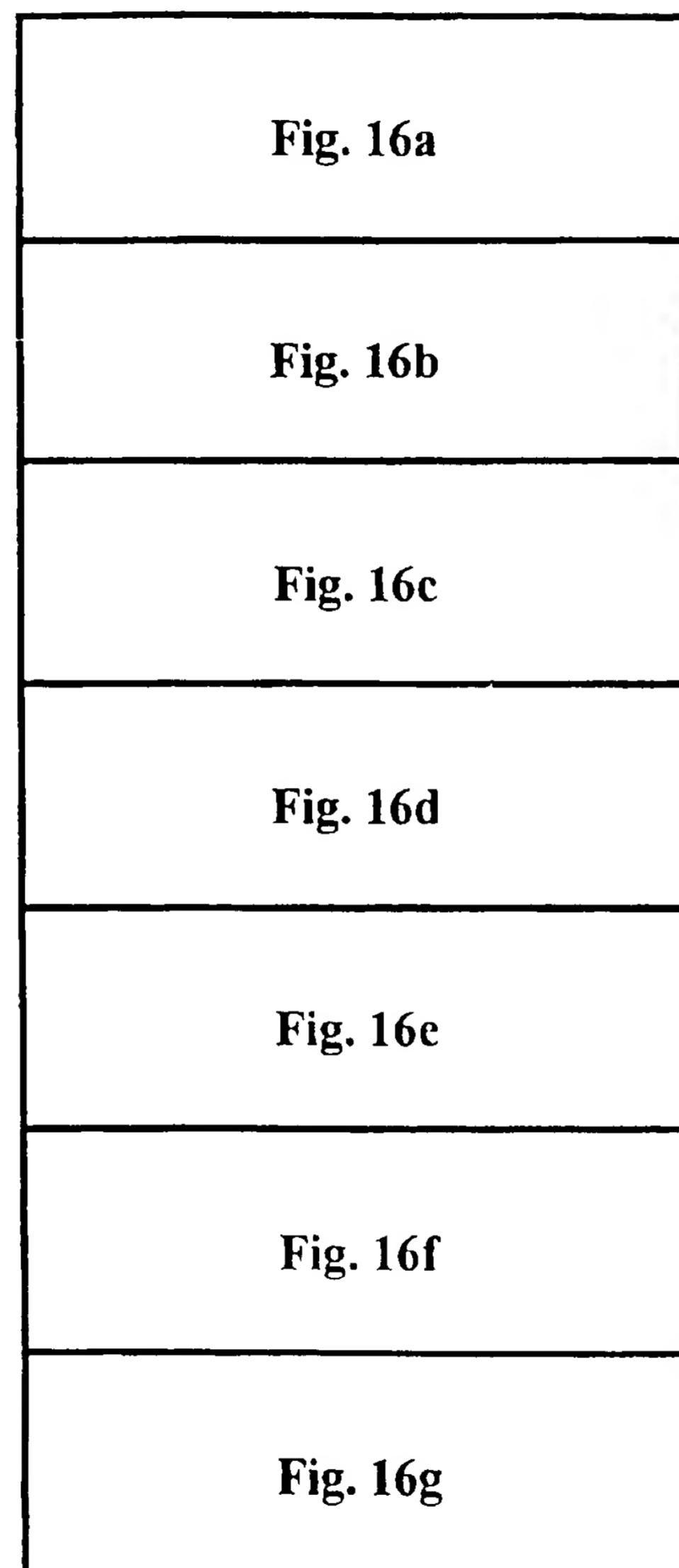
Fig. 15f

From Fig. 15b

To Fig. 15a

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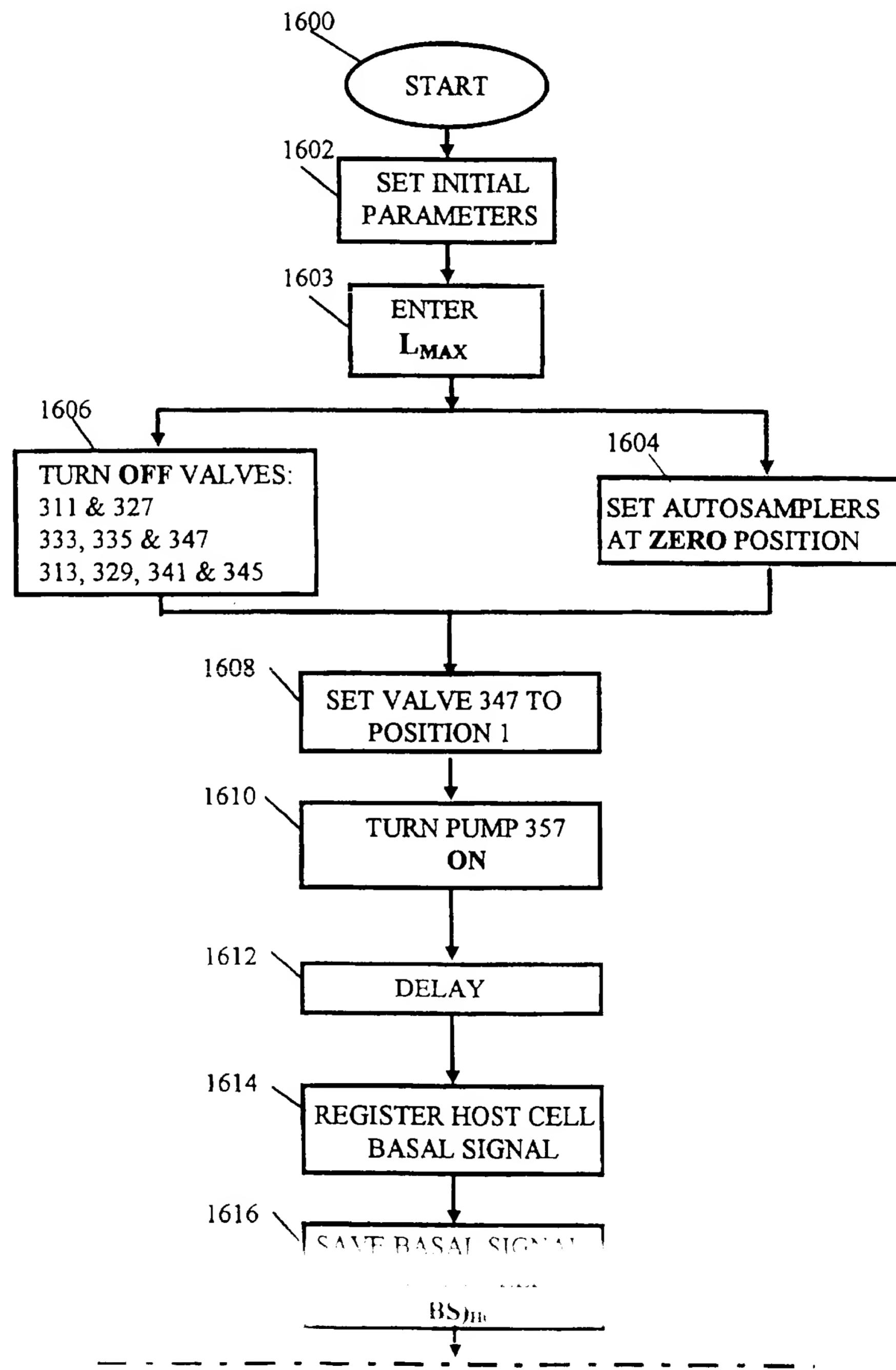
Fig. 16



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20

Fig. 16a



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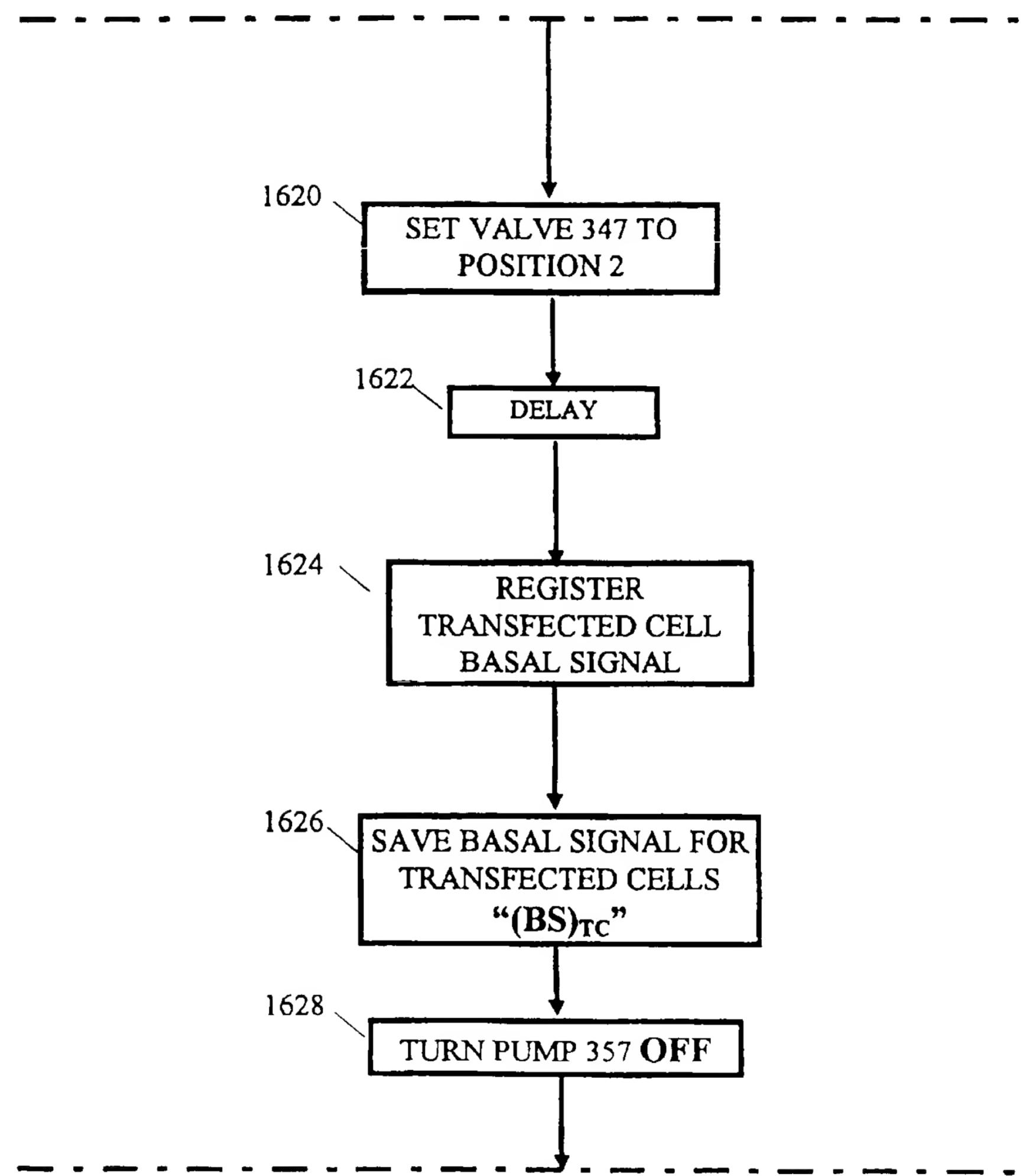
Fig. 16b

Fig. 16c

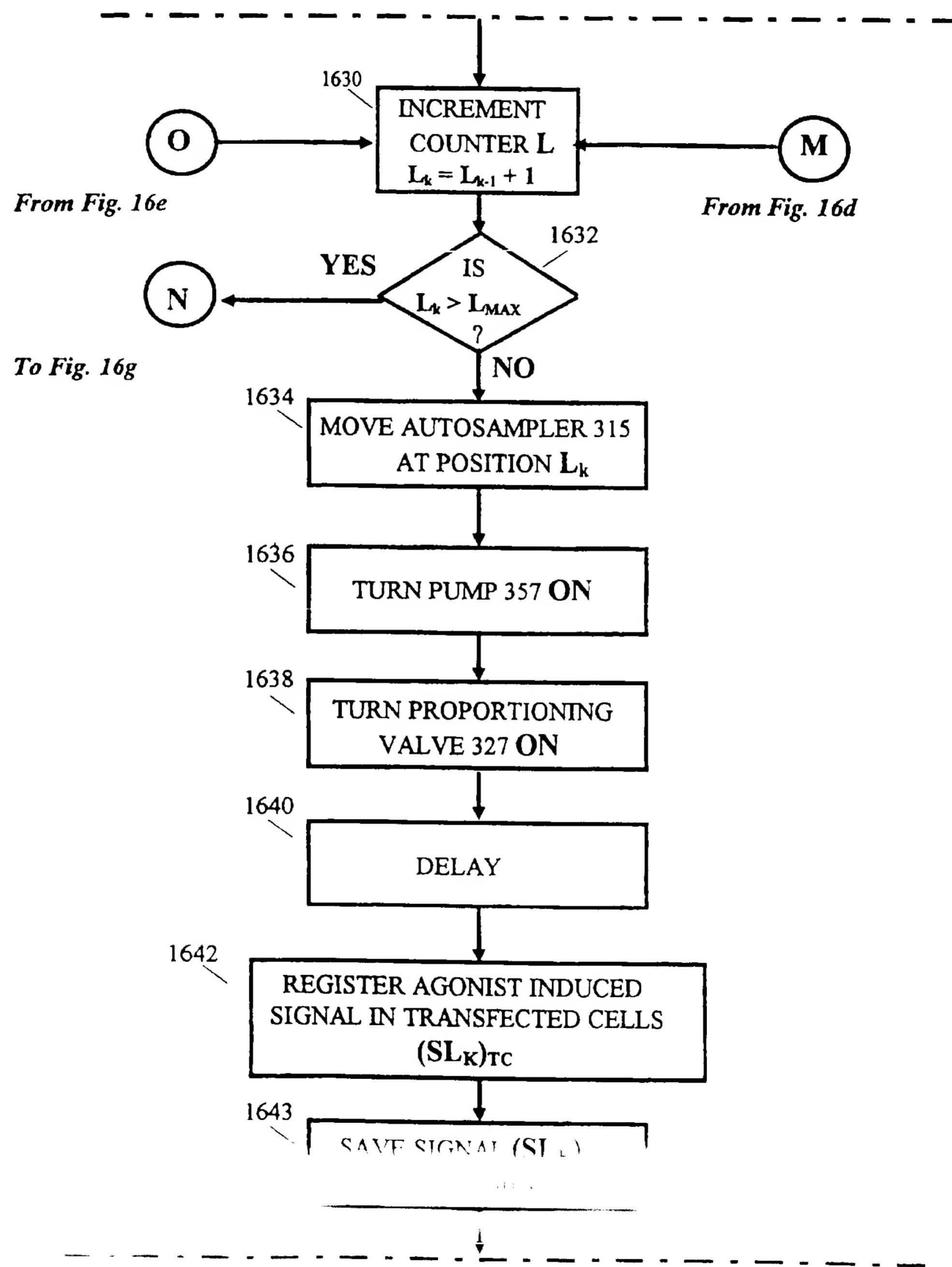


Fig. 16d

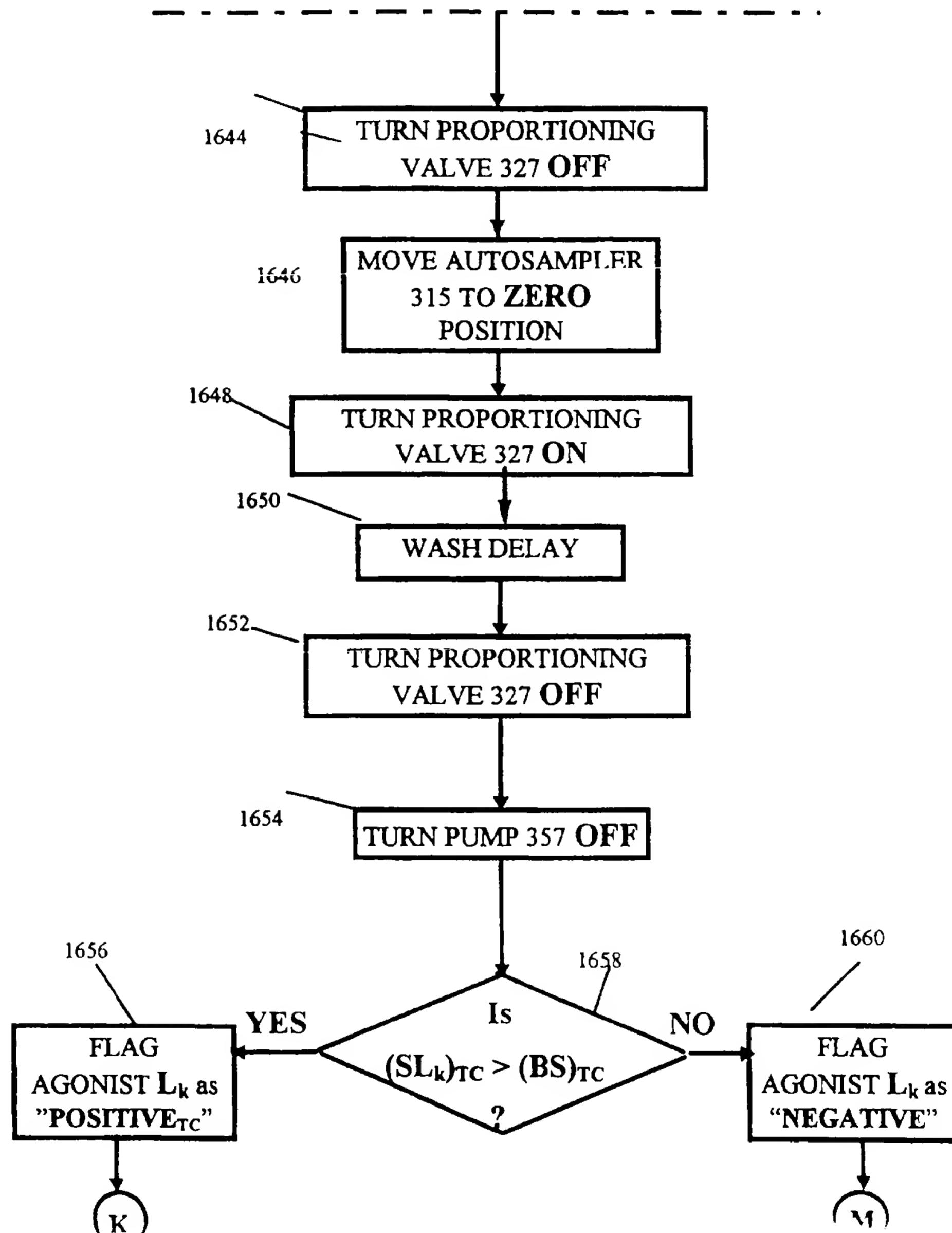


Fig. 16e

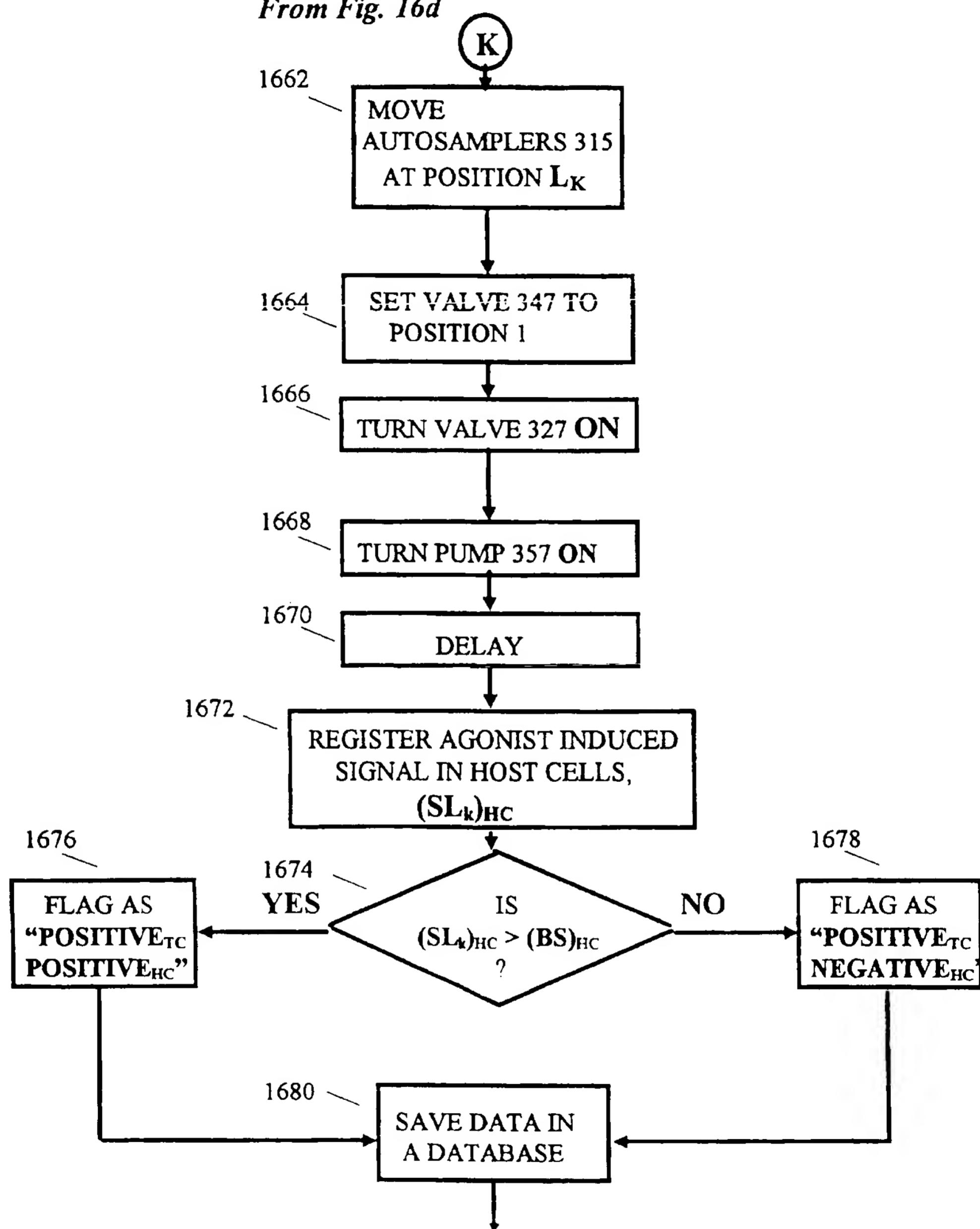
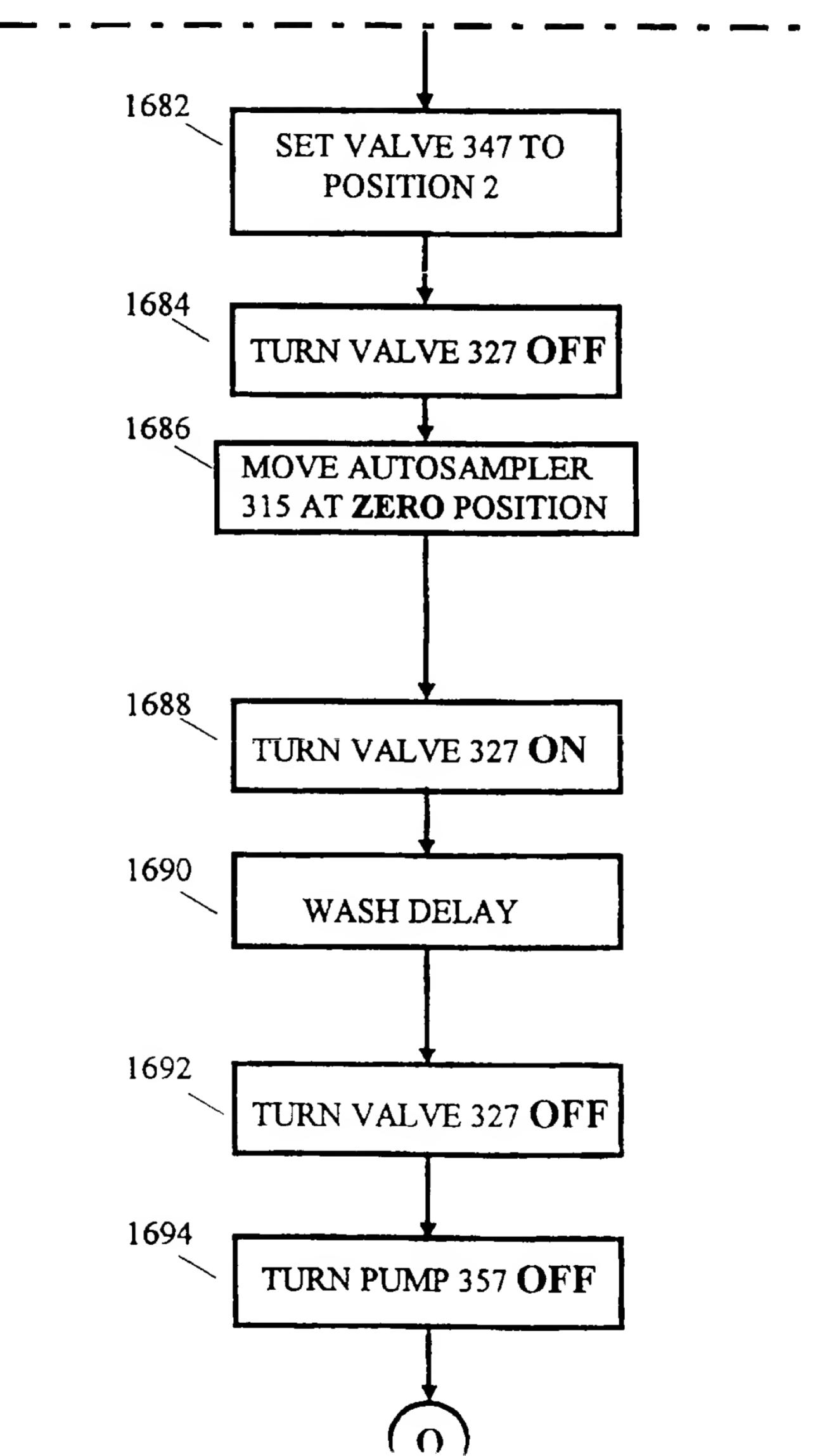
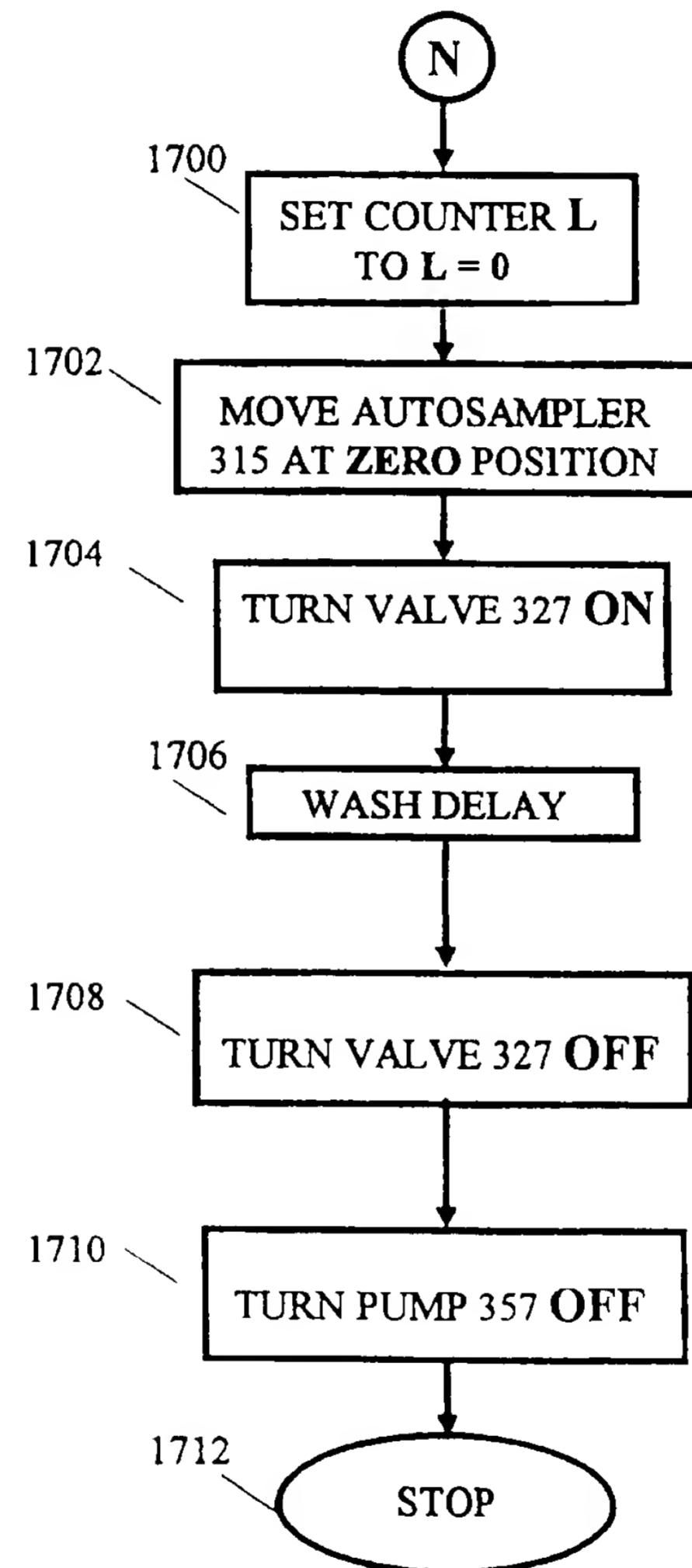
From Fig. 16d

Fig. 16f



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Fig. 16g*From Fig. 16c*

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/13602

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 C12M3/02 G01N35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 96 30760 A (NOVONORDISK AS) 3 October 1996 see claims 1-8 ---	1-81
X	WO 93 13423 A (SALK INST BIOTECH IND) 8 July 1993 see claims 1,15,20,31,42,44,46-87 see page 31, line 20 - page 38, line 30 see page 40, line 3 - line 10 ---	1-81
X	WO 90 04645 A (MOLECULAR DEVICES CORP) 3 May 1990 see claims 1-41 see page 3, line 8 - line 23 see page 5, line 30 - page 6, line 13 ---	1-81

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Patent family members are listed in annex.

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Date of actual completion of the international search

Date of mailing of the international search report

21 November 1997

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International Application No
PCT/US 97/13602

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 343 782 A (SHAPIRO HOWARD M) 10 August 1982 see claims 1-51 see column 8, line 13 - line 66 ----	1-81
X	US 4 271 123 A (CURRY ROBERT E ET AL) 2 June 1981 see claims 1-51 -----	1-81

INTERNATIONAL SEARCH REPORT

...Information on patent family members

Internat	Application No
	PCT/US 97/13602

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9630760 A	03-10-96	AU 5143096 A	16-10-96
WO 9313423 A	08-07-93	AU 3416893 A CA 2121506 A EP 0617791 A JP 7505710 T US 5670113 A	28-07-93 08-07-93 05-10-94 22-06-95 23-09-97
WO 9004645 A	03-05-90	AT 121790 T CA 2001212 A DE 68922390 D DE 68922390 T EP 0394406 A JP 3502642 T US 5496697 A US 5278048 A	15-05-95 21-04-90 01-06-95 05-10-95 31-10-90 20-06-91 05-03-96 11-01-94
US 4343782 A	10-08-82	BE 84 T CA 1132890 A CH 639776 A DE 2953524 A EP 0005032 A FR 2467402 A GB 2097530 A,B JP 1052699 B JP 1566300 C JP 54151895 A NL 7915051 A NL 7915051 T SE 455343 B SE 8008648 A ZA 7901851 A	19-09-80 05-10-82 30-11-83 06-08-81 31-10-79 17-04-81 03-11-82 09-11-89 25-06-90 29-11-79 28-11-80 28-11-80 04-07-88 02-12-80 29-10-80
US 4271123 A	02-06-81	NONE	

INTERNATIONAL SEARCH REPORT

Int'l. Appl. No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N15/14

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 11841 A (QUATRO BIOSYSTEMS) 26 May 1994 see page 1, paragraph 2 see page 8, line 5 - line 11 see page 9, line 9 - line 20 see page 11, line 5 - line 10 see page 11, last paragraph see figures 1-3	1-5
Y	---	10-12, 16, 18-24, 30-37, 43
	-/-	

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/03701

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 548 661 A (PRICE) 20 August 1996 see abstract see column 4, line 2 - line 34 see column 9, line 1 - line 58 see column 19, line 62 - column 20, line 30 see column 22, line 56 - column 23, line 40; figures 1,2 ---	10-12, 16, 18-24, 30-37,43
X	US 5 355 215 A (SCHROEDER) 11 October 1994 see column 1, line 23 - line 51	1-5
A	see column 6, line 26 - line 52 see figure 6 ---	10, 18-24, 30-37,43
A	WO 87 02802 A (CELL ANALYSIS SYSTEMS) 7 May 1987 see abstract see page 13, line 13 - line 22 see page 13, line 34 - line 37 see page 14, line 19 - line 21 see page 14, line 27 - line 37 see page 26, line 6 - line 37 see figures 1,11-14 -----	1,4,5, 10, 18-24, 30-37,43

INTERNATIONAL SEARCH REPORT

Information on patient family members

International Application No.

PCT/US 98/03701

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9411841	A 26-05-1994	NONE	
US 5548661	A 20-08-1996	NONE	
US 5355215	A 11-10-1994	NONE	
WO 8702802	A 07-05-1987	US 4741043 A 26-04-1988 DE 3688330 A 27-05-1993 DE 3689856 D 30-06-1994 DE 3689856 T 08-09-1994 EP 0245466 A 19-11-1987 EP 0248840 A 16-12-1987 EP 0571053 A 24-11-1993 JP 2527536 B 28-08-1996 JP 7168102 A 04-07-1995 JP 7146289 A 06-06-1995 JP 7181179 A 21-07-1995 JP 7111425 B 29-11-1995 JP 63501597 T 16-06-1988 US 4887892 A 19-12-1989 WO 8702803 A 07-05-1987 US 5109429 A 28-04-1992 US 5008185 A 16-04-1991 US 5016283 A 14-05-1991 US 5541064 A 30-07-1996 US 5086476 A 04-02-1992 US 5485527 A 16-01-1996 US 5134662 A 28-07-1992 US 5272759 A 21-12-1993 US 5281517 A 25-01-1994 US 5018209 A 21-05-1991	